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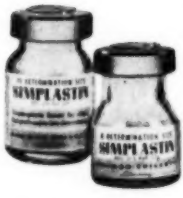

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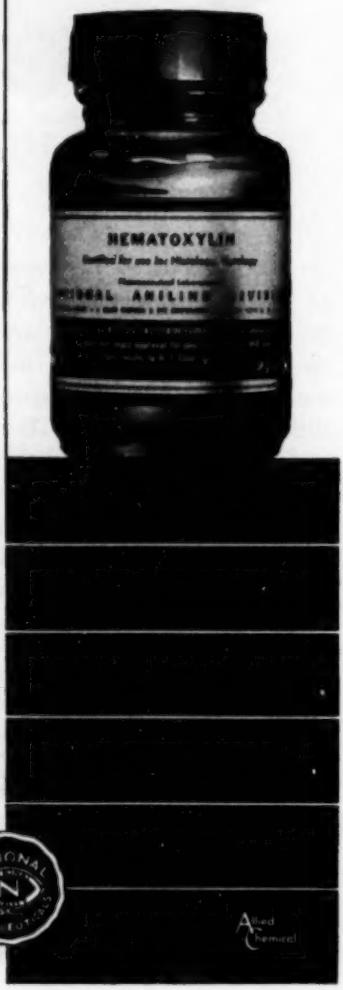

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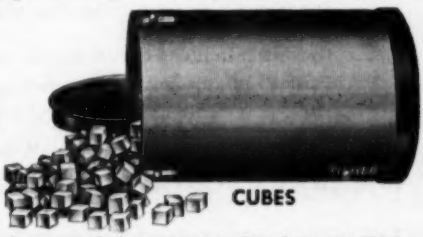

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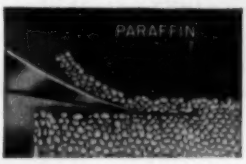

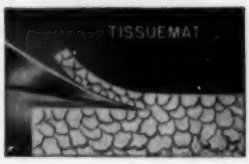

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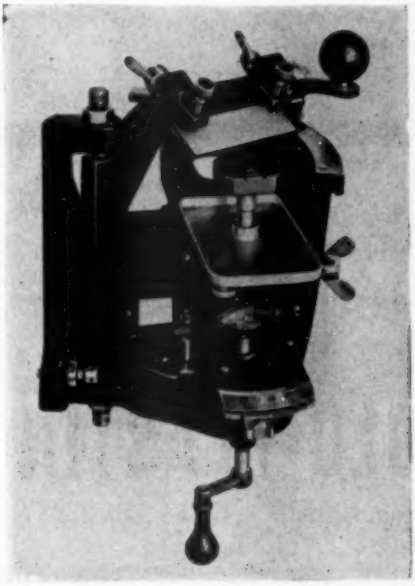

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VOLUME 58 JULY 1954 NUMBER 1

HISTOGENESIS AND BIOLOGIC BEHAVIOR OF GASTRIC CARCINOMA

Study of One Hundred Thirty-Eight Cases

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AND
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DENVER

CARCINOMA of the stomach has been evaluated by a strict or modified Borrmann's classification based on the amount of gross extension through the coats of the stomach,* by a modification of Dukes's method of grading depending on the microscopic spread through the coats of the stomach and on the presence or absence of metastases,† by Broders' method of grading on the basis of histologic differentiation,‡ by the presence or absence of metastases in regional lymph nodes,§ and by the histologic features in five-year survivors of gastric resection.[13] The most useful and accurate methods in the study of histogenesis and biologic behavior of the stomach include Dukes's method of grading [14] as modified by Dochat and Gray,[5] the presence or absence of metastases in regional lymph nodes and other sites as determined at autopsy or at autopsy in the postoperative period, and the histologic features as given by Steiner and co-workers.[13] Little progress could be made in solving the riddles of gastric carcinoma if all students of this disease would adopt the point of view expressed by the following quotation:

> Moreover I feel that it is not only a waste of time but also deceptive to attach histologically descriptive adjectives and prefixes to gastric carcinomas. Who is to determine the proportion of tubules a carcinoma shall form before it is to be called adenocarcinoma? How much mucin must be secreted in order that a tumor shall be called a colloid or mucoid carcinoma? Far more valuable is it to describe the gross forms of cancer, since it is important for diagnostic procedures to know these, since they also have some bearing on prognosis.

Even by using these gross forms as a basis for classification, about half of 82 gastric carcinomas resected in a period of five years were designated "carcinomas of no special type."[4] Granted that gross appearance of cancers elsewhere in the body as well as of gastric cancer may give strong clues as to their type, the microscope must be the final arbiter in the hands of the skilled pathologist for both classification and determination of prognosis. Since cancers of the thyroid gland, the breasts, the lungs, and the testes have certain growth patterns which denote a good or bad prognosis depending on the histologic features, cancer of the stomach should not be made an exception, as indicated by a previous study.[13]

From the Department of Pathology, University of Colorado School of Medicine.

* References 1-6.

† References 7 and 8.

‡ References 5, 7, and 8.

§ References 6 and 9-12.

MATERIAL AND METHODS

In the analysis of cases of carcinoma of the stomach accessioned in the Department of Pathology of the University of Colorado School of Medicine from June 30, 1927, through April 8, 1953, the material studied was assembled from June, 1951, through June, 1953 by one of us (R. R. R.) while a Student Fellow in Pathology during his third and fourth years of medical school. He was partly supported during this time by the Jim Fielder Cancer Fund of the University of Colorado and the Continuing Research Fund of the University of Colorado School of Medicine. When the preparation of a thesis based on this material for the M.S. degree became impractical because of the pressure of duties as a house officer in a local hospital, the desirability of a shorter communication to report these studies became apparent.

The minimal criteria for acceptance of the 138 cases of gastric carcinoma studied were the following: sufficient tissue sections of primary tumor as obtained at autopsy or by partial or complete gastric resection with regional lymph nodes attached to operative specimen (more numerous in the past 6 years than in the preceding 20 because of more extensive resection), sufficiently good preservation of primary tumor and metastases, adequate gross description of primary tumor and metastases, and proof of primary origin in the stomach. About 25 additional cases were rejected for the following reasons: Only tissue available was a small biopsy specimen of primary tumor or metastases, abdominal or distant; gross description was inadequate; autopsy tissues were poorly preserved; carcinoma was metastatic from other primary sites, two being small primary bronchial carcinomas, and tissue available for microscopic study was inadequate, even though the gross description was acceptable.

Most autopsy tissues studied were fixed in Zenker's fluid, and all surgical specimens were fixed in 4% aqueous formaldehyde solution. For the first 19 years covered by this study, the tissues were embedded in paraffin by the chloroform technique; for the last 7 years, by the dioxane technique. Sections were 6 to 8 μ thick. All tissues were stained with hematoxylin and eosin.

In 59 cases, selected blocks, usually of the primary carcinoma or occasionally of metastases, were stained by the periodic acid-Schiff routine, Mayer's mucicarmine method, and the Best glycogen technique. Most of these specimens were obtained at operation, and a few at autopsy, within a few hours of death, thus insuring good preservation.

HISTOGENETIC CONCEPTS OF GASTRIC CARCINOMA

In order to understand the presentation of the data of 138 cases of gastric carcinoma, a few introductory remarks concerning histogenesis are necessary. Theoretically, the mucous cells of the entire surface and upper part of the gland crypts of the gastric mucosa, the mucous neck cells, the pyloric and cardiac gland cells deep in the mucosa of the antrum and cardia, the parietal and chief cells of the gland crypts of the fundic mucosa, metaplastic intestinal epithelial cells, Paneth cells associated with metaplastic intestinal epithelial cells, metaplastic squamous epithelial cells, and heterotopic pancreatic ducts, acini, or islets could be progenitors of carcinoma of the stomach. The mucous cells and mucous neck cells of the stomach should probably be considered together as sources of gastric carcinoma, since no evidence to the contrary has appeared, nor was any forthcoming in the present study to lead one to believe otherwise. Evidence for mucous cells as a frequent source for gastric carcinoma has been presented.‖ Little emphasis has been placed on the pyloric and cardiac gland cells as precursors of carcinoma, but the histologic features of some carcinomas of Group III of McPeak and Warren [20] suggest such an origin. The role of metaplastic intestinal epithelial cells in forming gastric carcinoma has been doubted [16] or minimized,[4] although descriptive and pictorial evidence has favored this cell as an important source of gastric carcinoma, whether developing in polyps

‖ References 4 and 19.

or as a "superficial spreading" growth ¶ or when associated with pernicious anemia.# If carcinoma develops from the normal parietal or chief cells or from the Paneth cells associated with metaplastic intestinal epithelium, no proof for this has been found in the literature or in the present study. We have discarded the notion, appearing in abstract [26] and presented in preliminary form on several occasions without publication, that a type of histologically undifferentiated, but biologically relatively restrained, carcinoma of the stomach arises in the chief cells. We now feel, as will be more fully elaborated, that this type originates in metaplastic intestinal epithelial cells and is the "blue cell cancer" of Steiner and co-workers.[13] The occurrence of adenoacanthoma and squamous cell carcinoma of the stomach from metaplastic squamous epithelial cells has been described.[17] A few references have been made to carcinoma springing from pancreatic heterotopia in the stomach.[18] This is a rare occurrence; apparently the carcinoma develops from either pancreatic ducts or islets. A single carcinoma in the present series probably arose in pancreatic ducts heterotopic in the stomach and is included in the five cases of unclassified carcinoma to be described. The view originally held by us [26] that the ducts of heterotopic pancreas account for a small but significant group of gastric carcinomas has been abandoned.

From the histologic features of the 138 cases of gastric carcinoma in the present study, three major types emerged. The first type, mucous cell (MC) carcinoma, grows frequently as signet-ring cells with abundant to little mucin within and around cells, in undifferentiated fashion as single cells or clusters of cells, and in incomplete small-gland pattern, but proliferates uncommonly in complete small- or large-gland patterns. The second type, pylorocardiac gland cell (PGC) carcinoma, often forms complete small and large gland patterns, but infrequently shows undifferentiated sheets of cells and pseudo-signet-ring cells. The third type, intestinal cell (IC) carcinoma, displays two growth patterns showing crossover in some tumors or in two primary tumors in one case. The first pattern (IC-1) consists predominantly of complete large and small glands with admixture of sheets of undifferentiated cells in some tumors. The second pattern (IC-2) is a combination of sheets of undifferentiated cells and few complete small glands.

Of the 138 cases of gastric carcinoma, 65 were classified as mucous cell, 35 as pylorocardiac gland cell, and 33 as intestinal cell. Actually, 2 cases of intestinal cell carcinoma showed 2 primary tumors each, making a total of 35 primary carcinomas of this type. The remaining five cases were unclassified, four being possibly of pylorocardiac gland cell type and one probably originating in a heterotopia of pancreatic ducts.

OBSERVATIONS AND COMMENT

Sex and Age.—The data for sex and age of the 133 patients with 135 (Table 3) histologically classified carcinomas are summarized in Tables 1 through 4. The male to female ratio given in Table 1 for 65 cases of mucous cell carcinoma is close to that for the entire series of 133, whereas this figure is more than double for the 35 cases of pylorocardiac gland cell carcinoma and decreased for the 33 cases of intestinal cell carcinoma. The striking difference for the PGC group may indicate hormonal influ-

¶ References 2, 4, 13, 18, 19, and 21-25.

References 3, 22, and 25.

ences worthy of further exploration. The greater preponderance of females in the IC group is difficult to explain when the equal frequency of metaplastic intestinal epithelium in the sexes is considered.[16]

The minimum, maximum, and average ages for the three types of gastric carcinoma in Table 2 become more subject to analysis when the breakdown of these figures by decade in Table 3 is examined. The earlier age onset and decline of mucous cell carcinoma contrasts with the later occurrence and decrease of incidence with age for pylorocardiac gland cell and intestinal cell carcinoma. These figures suggest that carcinogens affecting the mucous cells begin to operate and to perform their deadly work earlier than those acting upon the other two varieties of cells. Careful analysis of patients affected by gastric carcinoma in the second and third decades [29] would probably show mucous cell carcinoma as the preponderant type. The later age

TABLE 1.—*Gastric Carcinoma, Sex*

	Males		Females		Total		
	No.	Per Cent	No.	Per Cent	No.	Per Cent	M:F Ratio
MC	51	78.5	14	21.5	65	50.0	3.64:1
PGC	31	88.5	4	11.5	35	26.0	7.75:1
IC	23	70.0	10	30.0	33	24.0	2.30:1
Total	105		28		133		3.75:1

TABLE 2.—*Gastric Carcinoma, Age*

	No.	Minimum	Maximum	Average
All Cases	133	32	91	64.0
MC	65	32	84	62.0
PGC	35	45	91	66.6
IC	33	41	85	65.5

TABLE 3.—*Gastric Carcinoma, Age*

	Total No.	31-40 No.	31-40 Per Cent	41-50 No.	41-50 Per Cent	51-60 No.	51-60 Per Cent	61-70 No.	61-70 Per Cent	71-80 No.	71-80 Per Cent	81-90 No.	81-90 Per Cent	91-100 No.	91-100 Per Cent
MC	65	6	9.2	3	4.6	14	21.5	30	46.1	10	15.3	2	3.1	—	—
PGC	35	—	—	2	5.7	8	22.9	12	34.3	11	31.4	1	2.8	1	2.8
IC	33*	—	—	2	6.0	8	24.2	13	39.4	8	24.2	2	6.0	—	—

* Two primary carcinomas in two cases.

TABLE 4.—*Gastric Carcinoma, Sex and Age*

	Sex	No.	31-40	41-50	51-60	61-70	71-80	81-90	91-100
MC	M	51	4	2	9	23	11	2	..
	F	14	2	1	5	6	..	..	..
PGC	M	31	..	1	8	9	11	1	1
	F	4	..	1	..	3	..	..	..
IC	M	23	..	3	4	9	5	2	..
	F	10	..	..	3	4	3	..	..
Total			6	8	29	54	30	5	1
			4.5%	6.0%	21.8%	40.6%	22.6%	3.8%	0.8%

occurrence of pylorocardiac gland cell carcinoma seen in Table 3 is not readily explained. The similarity of growth patterns of this type of carcinoma to those of the endometrial glands during the menstrual cycle, which will be described in detail, suggests hormonal factors in etiology. More easily explained is the later age indidence of intestinal cell carcinoma, since metaplastic intestinal epithelium is more frequent with advancing age.[16] Hence, carcinogens would exert their effects in later decades, even though on a less differentiated type of epithelial cell than the mucous cell. The differences for age and sex are contrasted by decades in Table 4.

The male to female sex ratio of 3.75:1 for the 133 cases of gastric carcinoma approximates the 3:1 to 4:1 ratio given for most series reported from large centers.* The occurrence of 85% of the cases between 50 and 80 years of age (Table 4) is somewhat higher than the 70 to 80% given in other studies.† The male to female ratio for gastric cancer cases in 10 metropolitan areas of the United States in 1947 was 1.9:1.[39]

Site.—The histologic structure of the mucosa of the stomach will be used in the localization of the 135 gastric carcinomas classified, rather than the gross divisions often employed. In the present study, the normal mucous cells lining the entire

TABLE 5.—*Gastric Carcinoma, Site*

	Antrum		Fundus		Cardia		Entire Stomach	
	No.	Per Cent	No.	Per Cent	No.	Per Cent	No.	Per Cent
MC.....	38	58.5	15	23.1	3	4.6	9	13.8
PGC.....	22	62.6	6	17.1	7	20.0	..	....
IC.....	21	60.0	11	31.4	3	8.6	..	....

surface and upper part of the gland crypts of the mucosa of the stomach, the normal specialized cells lining the pyloric and cardiac glands deep in the mucosa of the antrum and cardia, and metaplastic intestinal epithelial cells found with increasing age throughout the gastric mucosa have been accepted as sources for most gastric carcinomas.

The occurrence of the majority of gastric carcinomas of this series (Table 5) within the antrum of the stomach is in accord with previous observations.‡ Mucous cell carcinoma involved the entire stomach in nine cases, but neither of the other two types of carcinoma was so extensive in any case. The high frequency of pylorocardiac gland cell carcinoma at the cardia is to be expected because of the concentration of the specialized cardiac glands deep in the mucosa. Why six carcinomas of this group grew preponderantly into the fundus is not clear, although such carcinomas could have originated in pyloric glands of the proximal part of the antrum and have presented as fundic growths. Eleven, or more than 30%, of the intestinal cell carcinomas were located in the fundus, six on the greater curvature or on the anterior or posterior walls, three on the lesser curvature, and two with location not stated. In the present study, three patients with this type of carcinoma had pernicious anemia, two with the primary tumor on the posterior wall and one with the primary

* References 2, 10, 11, and 27-38.

† References 2, 10, 11, and 27-38.

‡ References 1, 2, 4, 7, 10, 25, 27, and 30.

tumor on the anterior wall of the fundus. Torgersen [25] noted that in patients with pernicious anemia and gastric carcinoma, the greater curvature of the fundus was a more frequent site for the primary tumor than in a series of gastric carcinomas with which pernicious anemia was not associated.

Size.—In Table 6 are given the figures for size, or greatest diameter in centimeters, of the 135 gastric carcinomas classified. Size was usually directly proportional to the amount of extension and to the number of metastases, which will be detailed in subsequent sections. This was particularly true for mucous cell carcinoma, but in the other two types of carcinoma, large size of primary tumor was not always an indication of aggressive behavior. In a study of the diameter of 682 gastric carcinomas,[40] 5.1% were 18 mm. or smaller, 3.8% were 18 to 25 mm., 5.9% were 25 to 30 mm., 4.0% were 30 to 35 mm., 4.4% were 35 to 40 mm., and 76.8% were 40 mm. and larger. The observations that 37, or 28%, of the carcinomas in our series were 50 mm. or smaller, and 98, or 72%, were larger than 50 mm. are in close

TABLE 6.—*Gastric Carcinoma, Size in Centimeters*

	2-5 Cm.		5-10 Cm.		10 Cm. and Larger	
	No.	Per Cent	No.	Per Cent	No.	Per Cent
MC	14	21.5	23	35.4	28	43.1
PGC	13	37.1	16	45.7	6	17.1
IC	10	28.6	19	54.3	6	17.1

TABLE 7.—*Gastric Carcinoma, Stage*

	Stage I		Stage II		Stage III		Stage IV	
	No.	Per Cent	No.	Per Cent	No.	Per Cent	No.	Per Cent
MC	..	...	..	...	4	6.2	61	93.8
PGC *	1	2.8	3	8.6	8	22.9	23	65.7
IC †	3	8.6	6	17.1	8	22.9	18	51.3

* Two incidental at autopsy.
† Three incidental at autopsy.

agreement. In the same study,[40] the diameter of 638 gastric ulcers was also measured; 79% were less than 18 mm., 92.3% were less than 25 mm., and none was more than 40 mm. That many clinically benign ulcerated lesions of the gastric mucosa within the size range of gastric ulcer are actually carcinoma has been demonstrated in two studies in which carcinoma was found in nearly 20% [41] and in 13% [42] of the resected lesions by histopathologic study. The work of Alvarez and MacCarty [40] showed that any lesion of the gastric mucosa more than 40 mm. in diameter is cancerous until proved to the contrary. Certainly any lesion more than 50 mm. in diameter must be suspect. A direct correlation has been made recently between size of gastric carcinoma and the presence or absence of regional lymph node metastases as determined by study of tissues obtained by radical gastrectomy.[12]

Stage.—A slight modification of the method used by Dochat and Gray,[8] which was based on Dukes's classification,[14] has been employed in the staging of the 135 gastric carcinomas classified (Table 7). These authors obtained a much sharper determination of five-year survival following gastric resection with Dukes's method

of grading than with Broders' method of grading. In our study, the microscopic extension of the carcinoma within and beyond the stomach and the presence or absence of metastases have determined the following four stages: Stage I, limited to the mucosa; Stage II, extended into the muscle coats; Stage III, extended to the serosa, and Stage IV, extended into adjacent structures and/or metastasized. The relatively more aggressive behavior of mucous cell carcinoma of the stomach can be appreciated as compared with that of the other two types. Only 6.2% of the mucous cell carcinomas were confined to the stomach, as compared with 33.3% of the pylorocardiac gland carcinomas and 48.6% of the intestinal cell carcinomas.

In 143 cases of gastric carcinoma observed at autopsy, Stout [4] found 10.5% restricted to the stomach. In 158 autopsied cases of carcinoma of the stomach, Hard-

TABLE 8.—*Gastric Carcinoma, Size and Stage*

		2-5 Cm.		5-10 Cm.		10 Cm. and Larger	
	Stage	No.	Per Cent	No.	Per Cent	No.	Per Cent
MC	I-III	1	1.5	1	1.5	2	3.1
	IV	13	20.0	22	33.8	26	40.0
PGC	I-III	6	17.1	4	11.4	2	5.7
	IV	7	20.0	12	34.3	4	11.4
IC	I-III	5	14.3	9	25.7	3	8.6
	IV	5	14.3	10	28.6	3	8.6

TABLE 9.—*Gastric Carcinoma, Extension*

	53 MC	19 PGC	13 IC
Pancreas	20	2	..
Esophagus	8	4	1
Duodenum	6	2	..
Diaphragm	3	2	..
Liver	2	..	..
Spleen	..	1	..
Bile ducts	2	1	..
Colon	2	1	..
Total	43	13	1

ing and Hankins [10] noted that 28% of the tumors were confined to the stomach. In tabulating 711 carcinomas of the stomach, Walther [48] found 25% limited to the stomach, a figure the same as that observed in our series (Table 7).

Size and stage have been correlated in Table 8 by contrasting the size of carcinomas confined to the stomach, Stages I, II, and III, with the size of those spread beyond the stomach, Stage IV. These figures emphasize the remarks made in this and the preceding section.

Extension.—This was determined for 53 mucous cell carcinomas, 19 pylorocardiac gland cell carcinomas, and 13 intestinal cell carcinomas observed at autopsy or at surgery and autopsy within two months after operation. The number of extensions to adjacent structures is given in Table 9.

In Table 10, the extensions of each type of carcinoma have been listed, from none to four. By taking the total extensions and dividing them by the number of carcinomas in each group, a figure called the extension index (Ie) is obtained. The

tendency for intestinal cell carcinoma to invade locally is small, but pylorocardiac gland cell carcinoma is nearly as often extended beyond the stomach as mucous cell carcinoma.

Metastasis.—The metastases for the 53 mucous cell carcinomas, the 19 pylorocardiac gland cell carcinomas, and the 13 intestinal cell carcinomas observed at autopsy, or at surgery and autopsy within two months after operation, are listed in Tables 11 and 12. Regional lymph nodes § include those of the superior gastric, inferior gas-

TABLE 10.—*Gastric Carcinoma, Extension*

	0	1	2	3	4	To *	Tu †	Ie
MC	26	18	6	2	1	43	53	0.81
PGC	11	5	1	2	..	13	19	0.68
IC	13	1	..	..	..	1	13	0.08

* To, total extensions.
† Tu, tumors.

TABLE 11.—*Fifty-Three MC Gastric Carcinoma, Metastases*

Regional lymph nodes	31
Peritoneum	31
Abdominal lymph nodes	25
Liver	19
Lungs	18
Thoracic lymph nodes	12
Pleura	11
Adrenals	11
Intestine	10
Bones	9
Kidneys	6
Ovaries	5
Diaphragm	4
Esophagus	3
Peripheral lymph nodes	3
Spleen	2
Bladder	2
Uterus	2
Pericardium	2
Heart	2
Scrotum, meninges, prostate, seminal vesicles, larynx, pancreas, ureter, thyroid, thymus, pituitary, gall bladder, skin	1 each
Total	220

tric, greater omental, lesser omental, subpyloric, paracardial, and pancreaticolienal groups. Abdominal lymph nodes include all other groups within the abdomen, especially periaortic. The mucous cell carcinomas followed a pattern of metastasis like that recorded by others ‖ in autopsied cases. In Table 13, the metastases of each type of carcinoma have been listed, from 0 to 16. By taking the total number of metastases and dividing them by the number of carcinomas in each group, a figure called the metastasis index (Im) is obtained. This index and the extension index have helped to quantitate the biologic behavior of the three types of gastric carcinoma. The striking propensity of mucous cell carcinoma to metastasize is obvious.

§ References 6, 9 through 12.
‖ References 1, 2, 4, 10, 27, and 43.

In patients who survived radical gastrectomy for carcinoma, Moore and co-workers [6] found that those with metastases in regional lymph nodes had a 5.3% five-year survival rate, as compared with those without metastases in regional lymph nodes, who had a 45% five-year survival rate. When the gastric carcinomas of these patients were typed by the Borrmann method, the patients with Types 1 and 2 showed an 18.7% five-year survival, whereas those with Types 3 and 4 showed a 10.9% five-year survival. Thus, in their experience, the presence or absence of metastases to regional lymph nodes was a much more accurate indicator of prognosis than the Borrmann method of typing.

Additional cases of pylorocardiac gland cell and intestinal cell carcinoma studied at autopsy or at surgery and autopsy within two months after operation would be

TABLE 12.—*Gastric Carcinoma, Metastases*

19 PGC		13 IC	
Regional lymph nodes	10	Regional lymph nodes	6
Peritoneum	6	Peritoneum	1
Liver	6	Liver	1
Abdominal lymph nodes	4	Abdominal lymph nodes	1
Thoracic lymph nodes	1	Thoracic lymph nodes	1
Lungs	2	Bones	1
Pleura	1	Adrenals	1
Intestine	1		
Spleen	1	Total	12
Total	32		

TABLE 13.—*Gastric Carcinoma, Metastases*

	No. Metastases													Total Metastases	Tumors	Im
	0	1	2	3	4	5	6	7	8	9	10	11	16			
MC	4	8	6	7	6	5	6	3	2	1	1	2	1	220	58	4.15
PGC	7	4	2	2	2	2	..	..	..	..	..	..	..	32	19	1.68
IC	6	4	2	..	1	..	..	..	..	..	..	..	..	12	13	0.92

desirable, but we feel that when these become available, the contrast between the biologic behavior of these two types of carcinoma and that of mucous cell carcinoma will be heightened.

Residual Fifty Cases of Gastric Carcinoma.—These 50 cases were not analyzed in the preceding two sections and include the following, as indicated in Table 14: 9 patients with autopsy beyond the two-month postoperative period, 28 dying two months or more after operation without autopsy, 11 treated surgically with postoperative follow-up, and 2 not traced.

A brief summary of the nine cases with autopsy beyond the postoperative period of two months is as follows:

CASE 39.—MC carcinoma; 20 months between resection and autopsy; male, aged 68; antrum tumor, lesser curvature, 4 cm.; Stage IV; metastases to peritoneum, intestines, pleura, and lungs.

CASE 52.—MC carcinoma; 14 months between resection and autopsy; male, aged 66; fundus tumor, over 10 cm.; Stage IV; metastases to peritoneum, intestines, bladder, diaphragm, thoracic lymph nodes, bones, and adrenals.

CASE 75.—PGC carcinoma; 14 months between resection and autopsy; male, aged 70; fundus tumor, greater curvature, 6 cm.; Stage IV; extended to colon and abdominal wall, no metastases; pernicious anemia.

Case 87.—PGC carcinoma; 13 months between resection and autopsy; male, aged 59; antrum tumor, 4 cm.; Stage IV; metastases to regional and abdominal lymph nodes, peritoneum, and intestine; jejunum perforated proximal to large metastasis with peritonitis.

Case 94.—PGC carcinoma; 15 months between resection and autopsy; male, aged 72; antrum tumor, 8 cm.; Stage IV; extended to pancreas, hepatic ducts, and liver; no metastases.

Case 98.—PGC carcinoma; 21 months between gastrojejunostomy and autopsy; female, aged 50; antrum tumor, 5 cm.; Stage IV; metastases to abdominal lymph nodes, peritoneum, intestine, left ovary, and to pseudomucinous cystadenoma of right ovary.

Case 112.—IC carcinoma; four months between resection and autopsy; male, aged 64; fundus tumor, 10 cm.; Stage IV; extended to esophagus; metastases to regional and abdominal lymph nodes and peritoneum.

Case 118.—IC carcinoma; 46 months between resection and autopsy; female, aged 79; antrum tumor, lesser curvature, 8 cm.; Stage IV; metastases to regional, abdominal, and thoracic lymph nodes, peritoneum, liver, intestine, bladder, lungs, adrenals, and cervical stump remaining after supracervical hysterectomy 52 years before death.

Case 126.—IC carcinoma; 10 months between resection and autopsy; male, aged 58; cardia tumor, 8.5 cm.; Stage IV; extended to esophagus; metastases to regional, abdominal, and thoracic lymph nodes, intestine, diaphragm, bladder, liver, pleura, lungs, thyroid, heart, kidneys, and adrenals.

Case 118 and Case 126 show that removal of the primary tumor may relieve obstruction and allow enough time for widespread metastases to occur before death.

Table 14.—*Residual Fifty Cases of Gastric Carcinoma*

	1*	2†	3‡	4§	Total
MC	2	9	1	..	12
PGC	4	9	3	..	16
IC	3	10	7	2	22
Total	9	28	11	2	50

* Autopsy beyond two-month postoperative period.
† Died two months or more after operation, no autopsy.
‡ Surgery and postoperative follow-up.
§ Not traced.

This is especially true of cardia carcinomas or of fundus carcinomas involving the esophagus, as the anatomic situation favors greater lymphatic and venous metastasis. Since the biologic behavior of gastric carcinomas subjected to primary autopsy or to autopsy within two months after gastric resection cannot be properly compared with the biologic behavior of gastric carcinomas subjected to autopsy several to many months after operation, these nine cases subjected to autopsy 4 to 46 months after resection or gastrojejunostomy have been segregated from those analyzed in Tables 9 through 13.

Table 14 also shows that 9 cases (13.8%) of MC carcinoma, 9 cases (25.7%) of PGC carcinoma, and 10 cases (30.3%) of IC carcinoma were lost to more thorough study, because no autopsy was made.

In Table 15 are listed 37 patients with gastric carcinoma who died after surgery. Of those surviving 24 months or more after operation, one had a MC carcinoma, three had PGC carcinomas, and four had IC carcinomas, figures in themselves indicative of the slower lethality of the PGC and IC types of carcinoma than of the mucous cell type.

As of Nov. 1, 1953, 10 patients with gastric carcinoma treated surgically were alive and well. One patient with MC carcinoma was followed for 6 months

after operation; three with PGC carcinoma, 18, 49, and 68 months, and six with IC carcinoma, 18, 23, 26, 42, 46, and 64 months. An additional patient with IC carcinoma had clinical local recurrence at the gastrojejunostomy stoma 18 months after a "palliative" subtotal gastrectomy. Radical total or subtotal gastric resection would have offered him a much better chance for survival without disease.

Unclassified Carcinoma.—All five cases in this group showed well-differentiated glandular carcinoma, four possibly of pylorocardiac gland cell type and one probably originating in ducts of pancreatic heterotopia.[18] All five patients were males, 45 to 69 years of age, studied at autopsy. Three primary tumors were located within the antrum; two were located on the lesser curvature of the fundus. Three were 5 to 10 cm. in diameter, two were over 10 cm. Four were Stage IV; one was Stage III. All five carcinomas showed well-differentiated small and large glands. Two extended into the colon, one with the formation of a gastrocolic fistula. One perforated into the peritoneal cavity, caused peritonitis, and metastasized to regional lymph nodes and liver. Only one other showed metastases, and these were confined to the liver.

Atrophy of Gastric Mucosa and Pernicious Anemia.—To be regretted in this study of the types of gastric carcinoma was the inadequacy of routine sampling of grossly noncancerized mucosa in all cases to determine such phenomena as focal or

TABLE 15.—*Death from Gastric Carcinoma After Surgery, Thirty-Seven Cases*

	No. Cases	
MC	11	2, 5, 6, 9, 13, 14,* 16½, 18,† 20*, 21, 25 mo.
PGC	13	4 days; 3, 11, 13,* 14,* 14, 15,* 17, 18, 21,* 24, 28, 41 mo.†
IC	13	3 hours; 1, 1, 2, 5 days; 3,† 4,* 6, 10,* 34, 37, 41, 46 mo.*

* Autopsy.
† No clinical recurrence.

diffuse atrophy, denoted by the presence of variable amounts of metaplastic intestinal epithelium. Four patients of the entire series of 138 had pernicious anemia.¶ Three had coexistent intestinal cell carcinoma; diffuse atrophy of the noncancerous mucosa was proved in two cases by adequate sections. The fourth case of pernicious anemia had pylorocardiac gland cell carcinoma and proved atrophy of the noncancerous gastric mucosa.

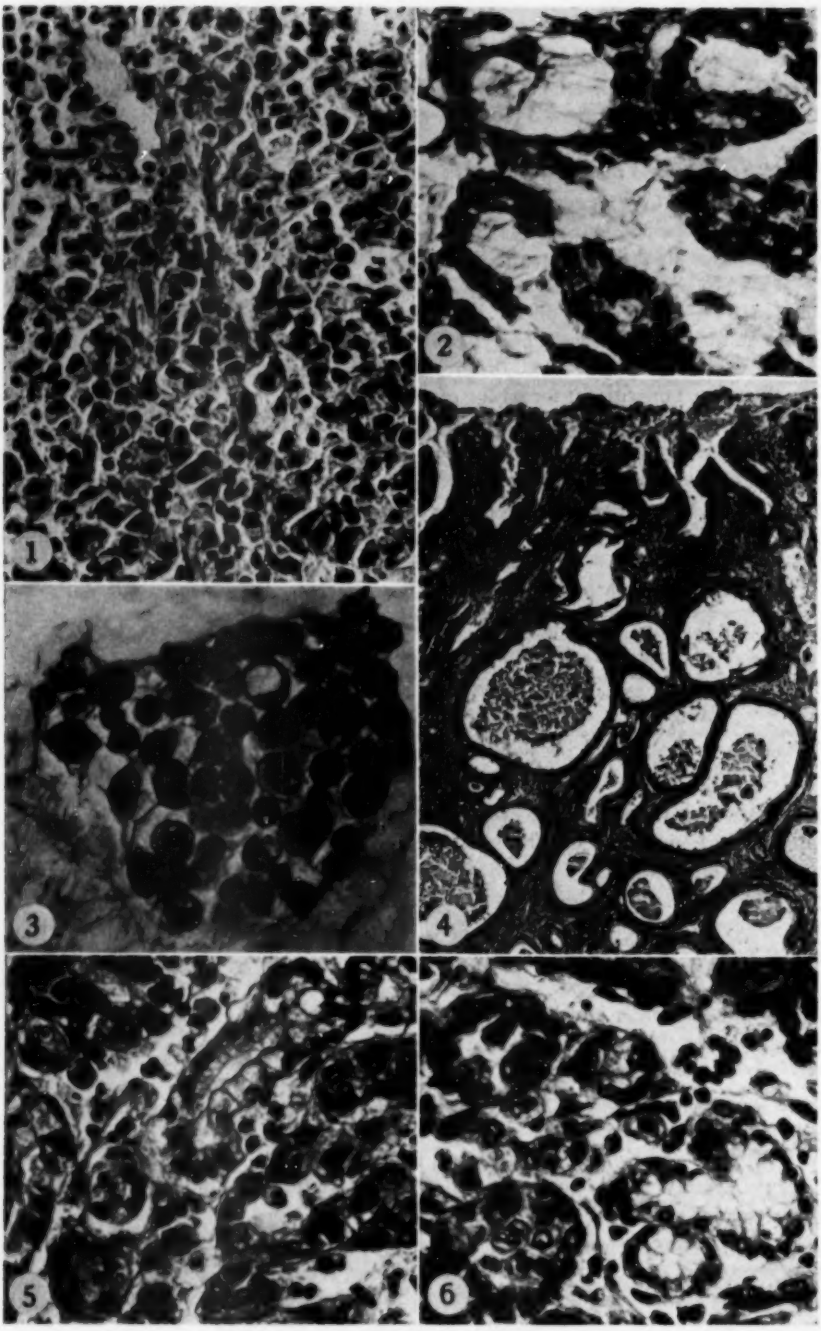
HISTOLOGIC SPECTRUM OF GASTRIC CARCINOMA

No more succinct statement could be found in the literature to express our quandary after the study of our first 25 cases of gastric carcinoma than that made by Benedict [9] as follows:

> But in cancer of the stomach the problem seems intricate and the idea of grading has been abandoned because of the different types of tumor encountered and the variation in structure in different parts of the same tumor.

After the first 65 cases of our series had been analyzed, some of the puzzling features of gastric carcinoma had been resolved,[26] and, as the foregoing sections dealing with 138 cases indicate, much progress has been made. Undoubtedly, study of 138 or more cases will vastly improve our attempts to evaluate the biologic behavior of gastric carcinoma by histologic pattern, as has already been accomplished for other cancers; namely, thyroid, mammary, bronchial, and testicular.

¶ References 22 and 25.



Figures 1-5

(See legends on opposite page)

The original stimulus toward analysis of the cases of gastric carcinoma accessioned in this department came from the relative paucity of information found in the literature for the correlation of histologic type and biologic behavior when a description of the histopathology of carcinoma of the stomach was attempted.[44] Fortunately, the text figures in Mulligan's "Syllabus of Human Neoplasms"[44] depict the various histologic types described in the present study. Figure 93 shows a mucous cell carcinoma, patient dead 25 months after operation with clinical recurrence, but no autopsy; Figure 96, a pylorocardiac gland cell carcinoma, patient dead of esophagopleurocutaneous fistula two months after operation, but no metastases at autopsy; Figure 95, an intestinal cell carcinoma of Type 1 histologic pattern, patient alive and well 42 months after operation, and Figures 92 and 94, intestinal cell carcinomas of Type 2 histologic pattern, patients alive and well 46 months and 36 months after operation.

TABLE 16.—*Gastric Carcinoma, Histologic Spectrum*

No.	Type	U *	SG †	SRC ‡	-Mucus	FLG §	LG ‖
65	MC	56	48	52	46	10	3
35	PGC	5	33	2	6	1	33
19	IC-1	5	19	0	1	1	16
16	IC-2	10	16	0	0	1	0

* Undifferentiated.
† Small glands.
‡ Signet ring cells.
§ Focal large glands.
‖ Large glands.

EXPLANATION OF FIGURES 1-6

Fig. 1 (Case 52).—Male, aged 66; fundus tumor, over 10 cm.; Stage IV; showing undifferentiated features, small gland pattern, signet-ring cells, and mucin. Autopsy, 14 months after radical total gastrectomy and esophagojejunostomy. Metastases involved abdominal, thoracic, and peripheral lymph nodes, peritoneum, intestines, pleura, and lungs. Tumor originating diffusely from gastric mucosa with barely perceptible origin from mucous cells and formation of cords and small tubules. Hematoxylin and eosin; × 400.

Fig. 2 (Case 49).—Male, aged 75; antrum tumor, 6 cm.; Stage IV; showing undifferentiated features, small gland pattern, signet-ring cells, mucin, and focal large gland pattern with metastases to regional lymph nodes. Died of clinical recurrence 21 months after operation. No autopsy. Area of serosa with imperfectly formed small glands suspended in intercellular mucin. Hematoxylin and eosin; × 400.

Fig. 3 (Case 40).—Male, aged 75; antrum tumor, 6 cm.; Stage IV; showing undifferentiated features, small gland pattern, signet-ring cells, mucin, and focal large gland pattern. Died of peritonitis four days after operation. Tumor extended to pancreas and metastasized to regional, abdominal, and thoracic lymph nodes, peritoneum, pleura, lungs, and adrenals. Muscle coats flooded by clusters of partly to completely formed signet-ring cells containing and surrounded by mucin. Hematoxylin and eosin; × 400.

Fig. 4 (Case 95).—Male, aged 59; antrum tumor, 7 cm.; Stage II; excised by radical total gastrectomy. Died 24 months after operation with signs of severe anemia, but without definite clinical evidence of recurrence. No autopsy. Lumen at top. Slightly autolyzed mucosa at surface. Neoplastic glands deep in mucosa, frequently dilated with flattened epithelium and extended into submucosa. Hematoxylin and eosin; × 35.

Fig. 5 (Case 70).—Male, aged 63; antrum tumor, 5 cm.; Stage IV; showing small gland and large gland patterns with metastases to regional lymph nodes. Death from peritonitis five days after limited subtotal gastrectomy. No additional metastases found at autopsy. Partly cancerous glands in upper right-hand corner. Anaplastic glands in remainder of field. Hematoxylin and eosin; × 400.

Fig. 6 (Case 70).—Note the few noncancerous epithelial cells in glands in lower right-hand corner; anaplastic cells in rest of field. Hematoxylin and eosin; × 400.

Mucous Cell Carcinoma.—The transformation of the normal mucous cells of the surface of the gastric mucosa and of the upper part of the gland crypts into carcinoma cells is often a barely perceptible and irregular process. In the grossly visible carcinoma, the involvement of the mucosa and other coats is diffuse and nondelimited. Most glands of the mucosa are cancerous, but portions of normal glands and even stretches of compressed noncancerous mucosa survive. The carcinoma cells have polyhedral, pale, acidophilic cytoplasm and enlarged, rounded nuclei with heavily stippled chromatin and minute or small nucleoli (Fig. 1). Mitotic figures are rare. Droplets of light basophilic mucin appear in the cytoplasm and coalesce until a single large globule displaces and flattens the nucleus to the periphery of the cell. This "signet-ring" cell is the hallmark of mucous cell carcinoma. The formation of signet-ring cells ranges from spotty to diffuse. The cells stuffed with mucin are frequently also suspended in mucin. The carcinoma cells are usually arranged in solid cords and small tubules on the persistent reticular framework of the mucosa. In a few carcinomas of this type, relatively perfect large and small glands in the mucosa are lined by cuboidal or cylindric neoplastic mucous cells and blend with more deeply

TABLE 17.—*Gastric Carcinoma, Special Stains**

	13 MC		22 PGC		12 IC-1		12 IC-2	
	I †	E ‡	I †	E ‡	I †	E ‡	I †	E ‡
PAS.....	238	185	164	245	...	150	17	160
	13 31	13 24	22 36	22 54	12 0	12 18	12 2	12 12
MuCa.....	225	200	24	152	...	75	9	18
	12 27	12 24	21 5	21 32	12 0	12 9	11 1	11 2
Glycogen.....	110	50	63	26	...	9	...	...
	10 11	10 5	19 12	19 5	11 0	11 1	10 0	10 0

* For explanation of Table see section Histologic Spectrum of Gastric Carcinoma, paragraph Special Stains.
† Intracellular.
‡ Extracellular.

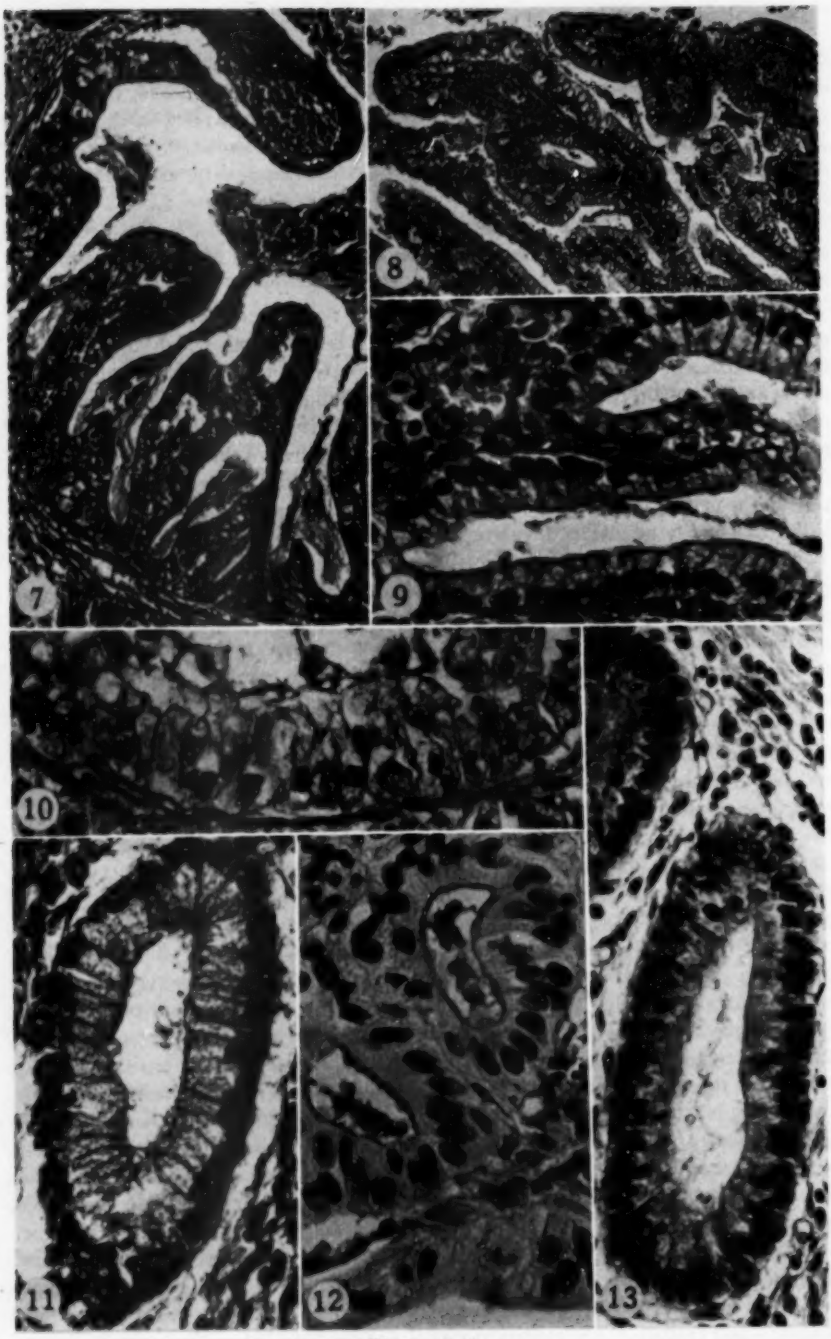
situated carcinoma cells like those already described. The infrequency of more perfect gland formation in the mucosa allowed only a few sections for study of this phenomenon. The only section which might have been used to depict this feature showed too much peptic digestion and acute inflammatory reaction to be suitable for a photomicrograph. With both the periodic acid-Schiff routine and Mayer's mucicarmine stain, the mucin of the normal mucous cells and of their cancerous counterparts was brilliantly stained, as was also extracellular mucin. In some mucous cell carcinomas, even glycogen stain tinges the intracellular material (Table 17). In the submucosa, muscle coats, and serosa, the carcinoma cells literally flood the connective tissue stroma (Fig. 3), move aside the smooth muscle bundles, encircle blood vessels, and invade lymphatics, including those around nerves. In these coats, the cells are dispersed in loose sheets, clusters, cords, or singly and are surrounded by variable amounts of increased collagenous connective tissue marked by lymphocytes.

The amount of fibrous connective tissue incited by the undifferentiated carcinoma cells seems to be indirectly proportional to the elaboration of mucin by them; the more mucin, the less is the connective tissue reaction; the less mucin, the greater is the productive fibrosis. Productive fibrosis is much more frequent and extensive in the extramucosal invasion of mucous cell carcinoma than in pylorocardiac gland cell or intestinal cell carcinoma. In the mucosa and other coats of the stomach, the

carcinoma cells also grow in solid interlocking sheets, punctuated by small rounded spaces and abortive signet-ring cells. Also observed are anastomosed, coiled cords of carcinoma cells forming incomplete small glands and suspended in mucin (Fig. 2). Formation of complete small glands is uncommon. Microscopic satellite foci of cancerized mucosa frequently appear in the grossly intact mucosa at the edge of the main carcinoma.[19]

In mucous cell carcinoma, as well as in the other two types, peptic digestion is associated with infiltration by segmented neutrophiles, which fill any glands present in the mucosa.

Pylorocardiac Gland Cell Carcinoma.—This type carcinoma usually grows in delimited fashion and fungates into the lumen or is sometimes widely ulcerated and acutely inflamed. One tumor invaded and perforated into the colon. Satellite foci adjacent to the main carcinoma are less frequent than in mucous cell carcinoma. The carcinoma cells originating in the pyloric and cardiac glands not only invade the submucosa, muscle coats, and serosa, but also spread toward the surface of the gland crypts and partly or completely displace mucous neck cells in the crypts and mucous cells in the crypts and at the surface (Figs. 5 to 12). As these carcinoma cells form both small and large glands, they are variably stratified or oriented in a single layer. The cells are low- to high-cylindric. When they are predominantly stratified, the cytoplasm is finely granular and acidophilic to partly clear. The central, parabasal, or basal ovoid or rounded nuclei contain lightly to heavily stippled chromatin and small nucleoli (Figs. 4 to 7, and 12). Clear areas may occur both above and below central or parabasal nuclei. Abnormal mitotic figures are relatively frequent. Secondary small glands are formed within or at the border of large glands. When oriented mainly in a single layer, the cells show partly clear to practically clear cytoplasm and rounded or ovoid basal nuclei with abundant finely stippled chromatin, small nucleoli, and relatively infrequent abnormal mitoses (Figs. 8 to 11). The basal nuclei are sometimes flattened perpendicular to the long axis of the cell (Fig. 11) to mimic the normal pyloric and cardiac gland cell. Transitions are noted between stratified and simple columnar patterns in the same and in different tumors. The neoplastic epithelial cells are arranged in undulated or papillary folds, especially in the stratified pattern (Fig. 7). The epithelial cells whether stratified or not may become so flattened by inspissation of secretion (Fig. 4) that the rounded spaces so formed mimic endothelial channels or spaces lined by mesothelium. This feature may be confusing in metastases. With the periodic acid-Schiff routine, the cytoplasm of the singly oriented cells contains more abundant positive material than the more granular or less clear stratified cells. The mucicarmine stain is infrequently positive for the material in these cells whether stratified or oriented singly. Even when positive, only a moderate reaction is obtained (Table 17). Normal pyloric and cardiac gland cells stain brilliantly with the periodic acid-Schiff (PAS) routine, but only moderately with mucicarmine stain, indicating close parallels between normal and cancerous pyloric and cardiac gland cells. The cells of occasional pylorocardiac gland cell carcinomas stain well with the glycogen technique. In many carcinomas of this type, material in the lumen of the glands shows a brilliant reaction with both PAS and mucicarmine stains. In a few, similar material surrounds some glands. In a few areas of a few carcinomas, the cells desquamate into the lumen of the glands, become rounded, and contain PAS-positive material. These have been called pseudo signet-ring cells.



Figures 7-13

(See legends on opposite page)

Because of the interplay between stratification and single orientation of the carcinoma cells in a single tumor, or from tumor to tumor, a parallel between the growth of pylorocardiac gland cell carcinoma and that of the endometrial glands in the proliferative and secretory phases of the normal menstrual cycle is suggested.

Invasion of pylorocardiac gland cell carcinoma into submucosa, muscle coats, and serosa is accompanied by relatively little productive fibrosis, except when the tumor becomes ulcerated and inflamed.

Intestinal Cell Carcinoma.—This type of carcinoma presents grossly as a polypoid, a fungating, or a relatively flat, nodular, delimited growth. In six tumors, origin from a glandular polyp was unequivocal#; two of these tumors were associated with benign glandular polyp, one single and the other multiple. Origin of the carcinoma cells from metaplastic intestinal epithelium * is comparable to the development of adenocarcinoma from the hyperplastic epithelium of glandular polyps of the large intestine. The types of growth of intestinal cell carcinoma include the IC-1 pattern, the reproduction of small and large glands by the carcinoma cells, and the IC-2 pattern, the formation of large sheets of undifferentiated carcinoma cells marked by small glands associated with abundant lymphoid tissue and relatively little connective tissue reaction. Regardless of gross appearance, the crossover between these two patterns in a single tumor or in two tumors in a single case

References 18, 21, 23, and 28.

* References 15 and 16.

EXPLANATION OF FIGURES 7-13

Fig. 7 (Case 92).—Male, aged 64; cardia tumor, 6 cm.; Stage III; showing small and large gland patterns. Death from local recurrence 18 months after partial gastrectomy by thoracic approach. No autopsy. In operative specimen, tumor extended to serosa of stomach, but greater and lesser gastric and omental lymph nodes were negative. At top, junction between lower border of stratified squamous epithelium of esophagus and carcinoma. Pseudostratification, finely foamy cytoplasm, and frequently basal nuclei in tumor cells. Hematoxylin and eosin; × 100.

Fig. 8 (Case 90).—Male, aged 66; antrum tumor, 4 cm.; Stage IV; showing small and large gland patterns with metastases to regional lymph nodes. Clinical recurrence 11 months after operation. Death 28 months after operation with signs of local recurrence. No autopsy. Hematoxylin and eosin; × 100.

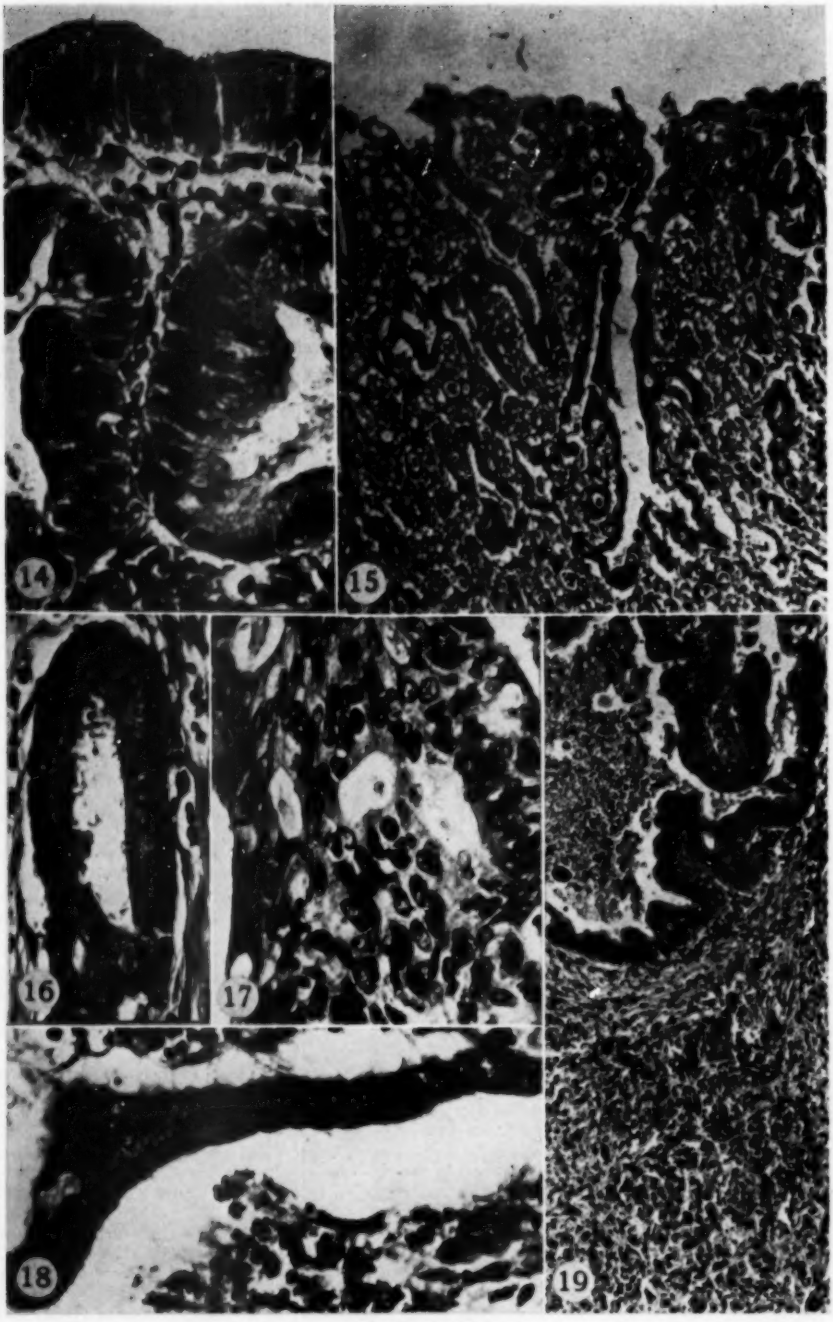
Fig. 9 (Case 90).—Enlargement of the gland in the lower right corner of Figure 8. Tumor involving all coats. Cells showing finely foamy cytoplasm, basal nuclei, and orientation in a single layer. Focal stratification in other fields. Appearance reminiscent of secretory endometrial glands. Lumen of glands frequently filled with purulent exudate. Hematoxylin and eosin; × 400.

Fig. 10 (Case 77).—Female, aged 64; antrum tumor, 4 cm.; Stage IV; showing small and large gland patterns, extension to common bile duct, and metastases to regional lymph nodes and liver. Cells finely foamy to partly clear, nuclei usually basal, local stratification. Appearance reminiscent of gestational endometrial glands. Metastasis in liver. Hematoxylin and eosin; × 400.

Fig. 11 (Case 89).—Male, aged 78; antrum tumor, anterior wall, 3 cm.; Stage IV; showing small and large gland patterns with metastases to regional lymph nodes at autopsy. Muscle coats containing neoplastic glands lined by columnar epithelial cells with finely foamy cytoplasm and basal nuclei. Hematoxylni and eosin; × 400.

Fig. 12 (Case 85).—Male, aged 72; antrum tumor, 7 cm.; Stage III; showing small and large gland patterns. Patient alive and well 68 months after radical subtotal gastrectomy. Cells finely granular to foamy, stratified, nuclei basal or parabasal. Hematoxylin and eosin; × 400.

Fig. 13 (Case 115).—Male, aged 70; known pernicious anemia for 10 years before subtotal gastrectomy for fundus tumor on anterior wall, 5 cm., polypoid; Stage I; showing small and large gland patterns. Alive and well 42 months after operation. Metaplastic intestinal epithelium lining glands at base of tumor and showing clusters of atypical nuclei, basal or parabasal. Cytoplasm finely granular and amphophilic. Hematoxylin and eosin; × 400.



Figures 14-19

(See legends on opposite page)

indicates the fundamental identity of the cell of both patterns as an anaplastic derivative of metaplastic intestinal epithelium. We believe that at least some, if not most, of the carcinomas in situ described by Mallory [23] and of the superficial spreading carcinomas defined by Stout,[24] whether peptically digested or not,[23] are actually intestinal cell carcinomas with a preponderance of IC-1 pattern. We also think that the blue cell cancer of Steiner and co-workers [18] is identical with the IC-2 pattern of intestinal cell carcinoma.

Metaplastic intestinal epithelial cells occurring in a polyp or replacing the normal cells of the surface and crypts of the gastric mucosa are low to tall cylindric have relatively homogeneous acidophilic cytoplasm, and contain ovoid, basal nuclei with finely stippled chromatin and small nucleoli. Single nuclei or clusters of nuclei are rounded, dyspolaric, marked by peripherally shifted chromatin, and distinguished by more prominent nuclei, features indicating atypicality. The cells are oriented singly or are stratified (Fig. 13). With the PAS routine and the mucicarmine stain, the lumen of these metaplastic glands and a few cells show moderately positive staining material. Glycogen stain is negative for both lumen and cells. The IC-1 pattern is denoted by increased stratification, flagrant dyspolarity of cells, progressively enlarged, rounded nuclei with irregular chromatin pattern, abnormal mitoses, and focal growth in small sheets, features which typify the transformation of metaplastic intestinal epithelial cells to carcinoma cells (Figs. 14 to 20, and 22 to 24). In the large anaplastic glands, the cells remain stratified or are singly oriented and may become flattened when the glands become dilated. Secondary small glands are formed within or at the margins of the large glands. The small glands proliferated by themselves reproduce a sinuous tubular arrangement and the cells are low columnar, cuboidal, or flattened. Clusters of segmented neutrophiles may be found in the lumen of the small or large glands, and lymphoid tissue varies in the connective tissue stroma, which is relatively sparse in the mucosa of the polypoid and flat lesions but is often increased as the carcinoma invades the submucosa, muscle coats, and

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EXPLANATION OF FIGURES 14-19

Fig. 14 (Case 115).—Detail of partly atypical and partly cancerous epithelium at surface and lining glands of the carcinoma. Left gland shows enlarged hyperchromatic nuclei, stratification, and disorderly cells. In other fields at base of tumor, anaplastic glands merged with sheets of undifferentiated carcinoma cells suggestively invaded into submucosa. Hematoxylin and eosin; × 400.

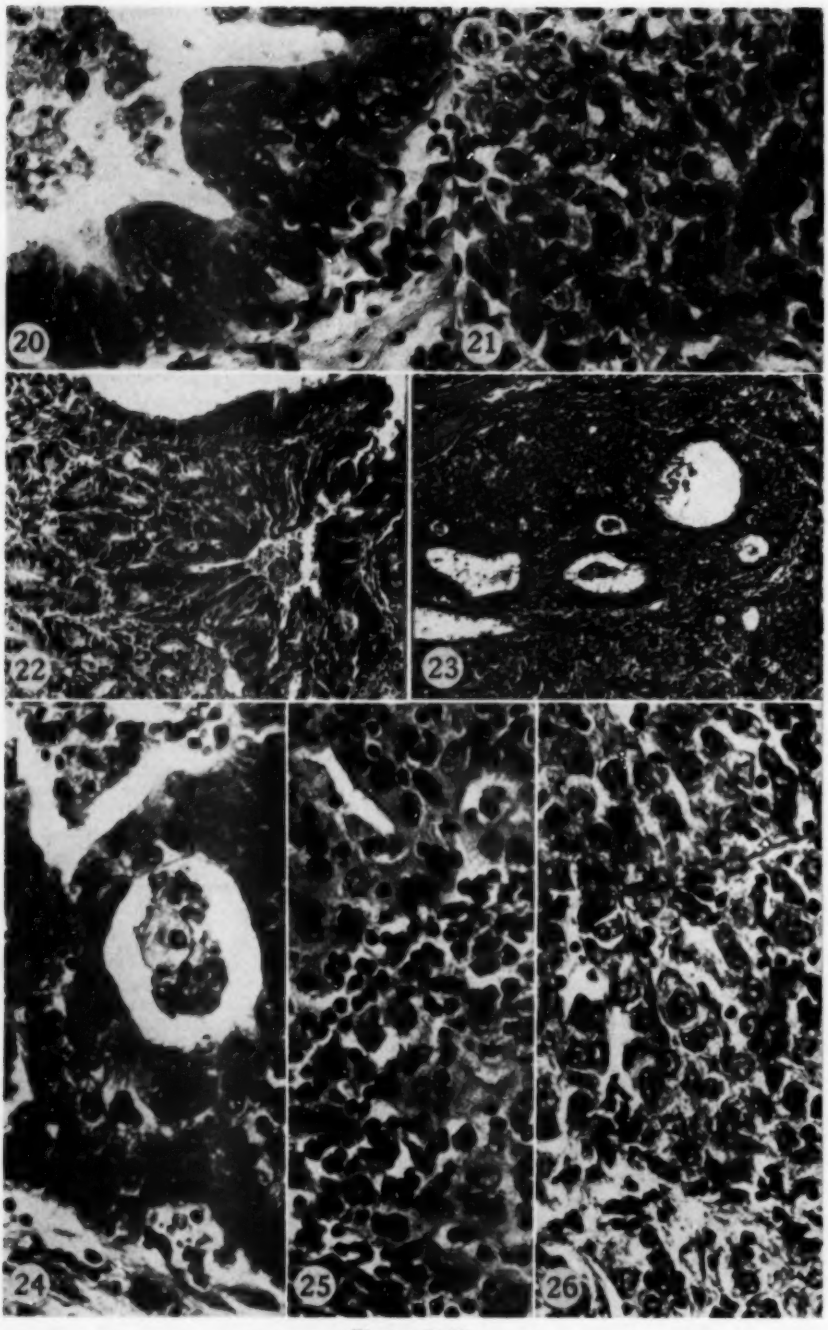
Fig. 15 (Case 106).—Female, aged 64; antrum tumor, lesser curvature, 3.5 cm.; Stage II; showing small and large gland patterns. Clinical recurrence 35 months and death 41 months following a limited subtotal gastrectomy. No autopsy. Radical subtotal gastrectomy would probably have cured this patient. Cancerous metaplastic intestinal epithelium in mucosa at surface and in crypts. Hematoxylin and eosin; × 100.

Fig. 16 (Case 106).—Transition of atypical metaplastic epithelial cells on left to carcinoma cells on right in gland of mucosa. Hematoxylin and eosin; × 325.

Fig. 17 (Case 106).—Few small glands and sheets of undifferentiated carcinoma cells in mucosa. Hematoxylin and eosin; × 325.

Fig. 18 (Case 106).—Detail of carcinoma cells lining gland of mucosa. Hematoxylin and eosin; × 325.

Fig. 19 (Case 104).—Male, aged 78, antrum tumor, lesser curvature, 4 cm.; Stage IV; showing undifferentiated features, small and large gland patterns, and origin from glandular polyp. At autopsy, mucosa of stomach marked by multiple glandular polyps, but metastases limited to regional lymph nodes. Large gland pattern in top half. Undifferentiated pattern and prominent lymphoid admixture in bottom half. Hematoxylin and eosin; × 100.



Figures 20-26

(See legends on opposite page)

serosa, especially when the tumor has become ulcerated and inflamed. With the PAS routine, only the material in the lumen of the anaplastic glands gives a moderate reaction. The same material is negative or slightly positive in a few cases with mucicarmine stain. The cells of the carcinoma are negative with both these methods. The glycogen stain is entirely negative. The same remarks for these stains apply to the lumen of the small glands and the cells of the IC-2 pattern (Table 17).

In the IC-2 pattern, the carcinoma cells are dispersed in compact to loose sheets with few small gland spaces interspersed, but with abundant intermingled lymphoid tissue (Figs. 19, 21, 23, 25, and 26). The cells are polyhedral, have amphophilic or pale basophilic cytoplasm, and inclose enlarged, rounded, or ovoid nuclei with heavily stippled, marginated chromatin, thick membranes, large nucleoli, and many bizarre mitotic figures (Figs. 21, 25, and 26). The interplay between both large and small glands of the IC-1 pattern and the undifferentiated sheets of carcinoma cells of the IC-2 pattern, the punctuation of these undifferentiated sheets of cells by small glands, and focal undifferentiated areas in the IC-1 pattern, all indicate a single carcinoma cell capable of growing as described and having growth characteristics comparable to those of adenocarcinoma of the large intestine.[45] The abrupt transition from mucosa transformed by metaplastic intestinal glands is another feature of intestinal cell carcinoma of the stomach, like that seen in adenocarcinoma of the large intestine. Regardless of IC-1 or IC-2 pattern, the prognosis is apparently equally favorable, whether one or the other is dominant in a given case. Somewhat remarkable also is the finding of two independent primary intestinal cell carcinomas in two of the 33 cases studied in this group, one with IC-1 pattern in both tumors, the other with IC-1 pattern in one tumor and IC-2 pattern in the other (Figs. 22 and 23).

EXPLANATION OF FIGURES 20-26

Fig. 20 (Case 104).—Detail of large gland pattern (Fig. 19). Enlarged, hyperchromatic nuclei in stratified, disorderly columnar cells arranged in undulated folds. Hematoxylin and eosin; × 400.

Fig. 21 (Case 104).—Detail of undifferentiated area (Fig. 19). Cells in loose sheets intermingled with lymphocytes. Nuclei enlarged, rounded, chromatin marginated, nucleoli prominent, abnormal mitoses. Hematoxylin and eosin; × 400.

Fig. 22 (Case 113).—Male, aged 65; first fundus tumor, posterior wall, 9.5 cm.; Stage IV; showing small and large gland patterns metastasized only to a superior gastric lymph node. Death from pulmonary embolism 13 days after radical total gastrectomy. No additional metastases found at autopsy. Large gland at top, large gland at right with many secondary glands, small glands at left. Hematoxylin and eosin; × 100.

Fig. 23 (Case 113).—Male, aged 65; second fundus tumor on anterior wall, 9 cm.; Stage IV; showing undifferentiated features and small glands, metastasized only to a superior gastric lymph node. Death from pulmonary embolism 13 days after radical total gastrectomy. No additional metastases found at autopsy. Sheets of undifferentiated carcinoma cells punctuated by small glands and surrounded by abundant lymphoid tissue. Hematoxylin and eosin; × 100.

Fig. 24 (Case 111).—Male, aged 81; antrum tumor, 3 cm.; Stage IV; showing small and large gland patterns and suggestive origin from glandular polyp. Death from severe staphylococcal pneumonia 35 days after limited gastrectomy. At autopsy, metastases confined to regional and abdominal lymph nodes. Merging of small gland pattern with undifferentiated areas. Hematoxylin and eosin; × 400.

Fig. 25 (Case 131).—Male, aged 41; fundus tumor, lesser curvature, 8 cm.; Stage III; showing undifferentiated features, small glands, and abundant lymphoid tissue. Alive and well 23 months after radical total gastrectomy. Hematoxylin and eosin; × 400.

Fig. 26 (Case 129).—Male, aged 71; antrum tumor, 8 cm.; Stage III; showing undifferentiated features in this field and spotty small gland pattern in others not depicted. Alive and well 46 months after radical subtotal gastrectomy. Histologic features identical with those in Figure 21. Hematoxylin and eosin; × 400.

A quantitative summary of the histologic features for the hematoxylin and eosin stain of all three types of carcinoma appears in Table 16.

Special Stains.—In Table 17 are given the results of the staining of 59 carcinomas, 13 mucous cell, 22 pylorocardiac gland cell, and 24 intestinal cell (12 with IC-1 pattern and 12 with IC-2 pattern), by the periodic acid-Schiff routine, the Mayer mucicarmine method, and the Best glycogen technique. All methods were used on formaldehyde- or Zenker-fixed tissues embedded in paraffin. The degree of staining reaction for intracellular and extracellular positive material for each type of carcinoma was graded; 0, none; 1, weakly positive; 2, moderately positive, and 3, strongly positive. The degrees of positivity for intracellular and extracellular material were totaled for each group of cases and each stain and an approximate index of tingibility was calculated. For instance, in the analysis of intracellular material of mucous cell carcinoma by the PAS routine, 13 cases were studied. The total of positive values in all 13 cases was 31. By dividing 31 by 13 below the line, the resultant figure 238 above the line gives a semiquantitative value for the whole group. If the value given above the line for each of the 24 sets of figures in Table 17 was 100 or more, this was arbitrarily taken as an indication that each stain was significantly positive. Probably the PAS routine and mucicarmine method would be sufficient to classify future cases. With the mucicarmine stain, a significant difference for the amount of positive intracellular material is seen between the mucous cell carcinomas, with a value of 225, and the pylorocardiac gland cell carcinomas, with a value of 24. A sharp separation of both patterns of intestinal cell carcinoma from mucous cell and pylorocardiac gland cell carcinoma is made by the practical absence of positive intracellular material with all three methods tried.

The PAS routine brilliantly tinged the cytoplasm of plasma cells and mast cells associated with all three types of carcinoma.

SUMMARY AND CONCLUSIONS

A study of 138 cases of gastric carcinoma resulted in the establishment of three major histologic types with characteristic biologic behavior; namely, mucous cell carcinoma, pylorocardiac gland cell carcinoma, and intestinal cell carcinoma.

Mucous cell carcinoma has the following features: frequent growth as signet-ring cells with mucin production or as undifferentiated cells, but uncommonly as differentiated glandular structures; significant onset before 40 years of age; primary tumor appearing as a relatively large, flat, diffusely infiltrative growth, and a high incidence of extension and metastasis.

Pylorocardiac gland cell carcinoma is characterized by the following: formation of well-differentiated glandular structures; significantly high incidence in males; onset usually after 40 years of age; localization preponderantly in antrum and cardia; primary tumor appearing usually as a delimited, fungating, sometimes widely ulcerated growth, and a diminished tendency for extension and metastasis as compared to mucous cell carcinoma.

Intestinal cell carcinoma displays the following traits: two growth patterns with interplay in some tumors or in double primary tumors, the first well differentiated glandular and the second mainly undifferentiated; a suggestively greater incidence in females than the other two types of gastric carcinoma; onset usually after 40

years of age; significant localization in the fundus; high frequency of pernicious anemia; primary tumor appearing as a polypoid, a fungating, or a relatively flat, nodular, delimited growth, with a low propensity for extension and metastasis.

Although all three types of gastric carcinoma extend, metastasize, and kill, the approximate degree of lethality as determined both at autopsy and by survival following gastric resection is as follows: mucous cell carcinoma 98%, pylorocardiac gland cell carcinoma 75%, and intestinal cell carcinoma 60%.

A valuable adjunct to the future study of gastric carcinomas, especially the pylorocardiac gland cell and the intestinal cell types, would be the application of elastic tissue stain to determine the relationship of vein invasion to survival and to the dissemination of metastases, notably to the liver.

Less radical gastric resection is probably indicated for mucous cell carcinoma, since prognosis is so poor even with the most radical total gastrectomy technically possible.

Depending on the case, radical gastrectomy of variable totality is indicated for pylorocardiac gland cell carcinoma and intestinal cell carcinoma. In patients with signs of local recurrence at the anastomosis site following gastric resection for either of these two types of carcinoma, an attempt at secondary resection seems warranted.

The similarity of the growth patterns of pylorocardiac gland cell carcinoma and of the endometrial glands during the menstrual cycle suggests the use of hormones, such as androgens or estrogens, in treatment of recurrent or metastatic carcinoma.

Further analysis of more cases of gastric carcinoma should be made by study of histologic features and biologic behavior to confirm or refute the observations described. This analysis should include the relationship between histologic type of carcinoma and location in the stomach to extension and metastasis.

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HOMEOSTASIS OF CONNECTIVE TISSUES

I. Calcium-Sodium Equilibrium

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GROUND substance of connective tissue consists largely of three types of component: colloids, diffusible electrolytes, and water.* Chemical characterization of the colloids depends on isolation of proteins, mucopolysaccharides, lipids, and other fractions from highly complex aggregates. Independently of the detailed characterization of its chemical composition, certain electrochemical characteristics of this organic matrix have been described. As an aggregate, the system of ground substance colloids constitutes an ionic exchange resin reacting with diffusible blood electrolytes. The electrochemical properties of this system are affected by hormones, such as relaxin and estrogen, in such a manner as to influence the distribution of water and electrolytes.†

Characterization of the inorganic part of the matrix depends on determinations of water and inorganic ions. This composition depends on the electrochemical properties of the colloidal matrix. One such measurable property is the density of the base- or acid-binding groups of the immobile colloids. This quantity may be estimated as equivalents per kilogram of tissue water. In the pubic symphysis of the guinea pig, the electrical charge density has been estimated, using the method of liquid junction potentials. It has been shown to be highly negative in the normal symphysis, where the tissue has a density approximating that of cartilage. In the relaxed symphysis, the negative charge density is low, and the water content of the tissue is high.‡ The apparent mobility of potassium is low in the normal tissue and high in the relaxed state. It has been concluded that the interaction of the charged colloidal residues with sodium and potassium favors selective adsorption of potassium in preference to sodium.

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* References 1 and 2.

† References 1 and 3.

‡ References 1 and 3.

In the present study, the electrometric method is applied to estimation of the calcium-binding properties of various connective tissues. The results lead to an estimation of equilibrium constants for these tissues; to estimations of ionic, bound, and total calcium for various states of connective tissue, and, finally, to an approximate nomographic description of the equilibrium among connective tissue, water, sodium, and calcium ions. Analytical figures relating inorganic salts to the water and colloidal contents of connective tissues are compared with the electrometric results, and are shown to yield similar values for a number of properties. In addition, the effects of parathyroid U. S. P. (parathyroid extract) have been studied. The state of ground substance colloids described electrometrically may be compared with histochemical observations on both normal tissues and on those modified by hormones.§

EXPERIMENTAL STUDY

The method of determination of junction potentials at boundaries of connective tissues and perfusing salt solutions has been described.[1] By means of saturated KCl-agar salt bridges, saturated KCl-calomel half-cells are connected to the reference and experimental tissue junctions. The reference junction of approximately isotonic (0.15 M) NaCl is applied subcutaneously in the abdominal region. An experimental junction is formed at the boundary of the tissue to be studied. Differences of potential are measured between the two indifferent electrodes.

The boundary that is studied may be represented by

Tissue | Solution I, II, or III

The potential difference at this experimental boundary is the variable part of the observed potential difference. It depends on the nature of the applied salt solution and on the electrochemical state of the tissue that is studied. The reference boundary is not varied. The displacements of the observed potential difference are readily reversible.

Solution I denotes approximately isotonic (0.15 M) NaCl; Solution II, a 1/10 dilution (0.015 M) NaCl, while Solution III refers to an approximately isotonic mixture of NaCl and $CaCl_2$ (0.135 M NaCl plus 0.01 M $CaCl_2$).

The effects are studied in the following sequence: First, the resting base line potential is observed with Solution I until three readings agree within 1 mv. Then the potential is displaced by substituting at the boundary Solution II for Solution I. The base line potential is then restored by again forming a boundary with isotonic NaCl and reequilibrating the tissue. Solution III is then substituted, resulting in only a slight change of boundary potential. The dilution potential is redetermined, and, finally, the base line potential is restored with Solution I.

The first displacement of the boundary potential obtained with Solution II is denoted by E_d, and is referred to as the "first dilution potential." The second displacement following substitution of Solution II for Solution III at the junction is denoted by E_d' and is referred to as the "modified dilution potential." E_d depends on the initial colloidal charge density at the tissue side of the experimental boundary. During equilibration with Solution III, a fraction of the negative colloidal charges appears to combine with calcium ions. As a result, the modified dilution potential, E_d', is considerably lower than the first dilution potential, E_d, and the difference is theoretically related to the quantity of calcium bound by the ground substance colloids.

Various types of connective tissues in monkeys and rabbits were studied. In the rabbit, these included the tibial epiphysis, abdominal skin, and gastrocnemius tendon; in the monkey, osteochondral areas in the sternum, dentin, gingiva, and skin. After normal values of the dilution potentials E_d and E_d' were obtained, the animals were given injections of parathyroid U. S. P.‖ in two doses over a 48-hour period. Rabbits received 1,000 units; monkeys, 1,000 to 1,500 units. At the end of a 48-hour period, potentials were redetermined on all tissues except rabbit tendon.

§ References 4 to 6.

‖ Parathormone (a parathyroid hormone) was supplied by Eli Lilly & Company, Indianapolis.

RESULTS

A summary of the results is presented (Table 1). In three dense tissues (sternum, dentin, epiphysis) the first dilution potential, E_d, was highly positive, about 20 mv., referred to the initial steady base line. After equilibration with Solution III, containing calcium, the modified dilution potential, E_d', fell, on the average, to slightly less than 10 mv. Effects of a parallel nature have been reported by Klein and Amberson [7] with potassium and calcium salts. If calcium was present in Solution III as either the citrate or the succinate, there was no observed effect on the dilution potential, E_d'. It was necessary to introduce calcium as the highly ionized chloride in order to observe the effect; combination of calcium with dicarboxylic or tricarboxylic acids evidently prevents its combination with connective tissue colloids. Parathyroid extract showed measurable effects on each of the dense tissues, decreasing E_d on the average by 4 to 7 mv. Rabbit tendon differed from the other dense connective tissues in showing negative dilution potentials (—8 to —12 mv.).

TABLE 1.—*Dilution Potentials in Normal and Parathyroid-Stimulated States**

Species	Tissue	Conditions†	No. of Experiments	First Dilution Potential E_d, Mv.	Modified Dilution Potential E_d', Mv.
Monkey	Skin	N	4	3.2	—2.9
		P	4	1.7	—4.1
	Sternum‡	N	10	23.2	7.2
		P	6	16.6	5.7
	Gingiva	N	7	8.2	—0.1
		P	6	1.3	—3.5
	Dentin	N	7	18.1	8.6
		P	5	14.0	5.4
Rabbit	Epiphysis	N	8	19.2	9.0
		P	8	12.7	3.3
	Skin	N	6	2.2	—3.4
		P	6	—1.2	—5.4
	Tendon	N	6	—10.4	...

* Values of dilution potentials are means for the group.
† N denotes normal state; P, stimulation by parathyroid extract.
‡ Osteochondral tissue.

Monkey gingiva is an example of softer connective tissue, for which the value of E_d was found to be about 8 mv. After equilibration with calcium, the modified dilution potential, E_d', was close to zero. Parathyroid extract showed strong effects in lowering these values to about 1 mv. and —3 mv., respectively. Monkey and rabbit skin showed initial dilution potentials of +2 or +3 mv., and modified dilution potentials of about —3 mv. Parathyroid extract had rather small effects on these values.

PHYSICOCHEMICAL CONSIDERATIONS OF THE DILUTION POTENTIAL

Theoretical and experimental relations between the observed boundary potentials and the electrochemical state of connective tissues have been presented.¶ Based on the thermodynamic studies of Henderson # and Donnan,[10] the treatment relates the observed dilution potentials to the concentration of immobile colloidal charges in the tissue. Thus the dilution potentials, E_d and E_d', depend on the free base-binding groups of the immobile colloids before and after equilibration with calcium ions.

¶ References 1 and 3.
# References 8 and 9.

The difference is a measure of the combined calcium. Relations between ion concentrations and colloidal charges are determined by the Gibbs-Donnan conditions for electrostatic and thermodynamic equilibrium. As first approximations, $(Na^+) = 0.15 + x/2$, and $Cl^-) = 0.15 - x/2$, where x is the density of immobile negative colloidal charges. The unit of charge density is taken as equivalents per kilogram of water in the colloidal system (or tissue). The two approximations become rather inaccurate at high values of x. Certain corrections are therefore necessary for dense connective tissues. The following mathematical treatment is based on derivations applied to the pubic symphysis of the guinea pig.[3] A detailed account has been presented in the review of Frieden and Hisaw.[11]

Since the colloid is immobile, current at the boundary is carried by sodium and chloride ions. The fraction n_1, carried by sodium ions, is given by the expression

$$n_1 = \frac{0.15 + 0.5x}{0.375 - 0.25x} \tag{1}$$

The fraction n_2, carried by chloride, is given by $(1 - n_1)$. The expression for n_1 has been derived from the Gibbs-Donnan values for (Na^+) and (Cl^-) by taking the relative mobility of sodium ion as 1.0 and that of chloride ion as —1.5. These values agree with standard electrochemical data. At values of x greater than about 0.06, n_1 is greater than 0.5; at smaller values of x, n_1 is less than 0.5. At high values of x, most of the current is carried by sodium ions, and the ground substance shows selective cation permeability.*

The dilution potential E_d depends on the displacement of the initial equilibrium boundary potential by substitution of 0.015 M NaCl at the junction. At 37 C. it has been shown to be given by the following expression:

$$E_d = 61.7\ (2n_1 - 1) \tag{2}$$

where the numerical factor depends on the temperature.

Substitution of the value of n_1 yields the approximation

$$E_d = -12.3 + 215x \tag{3}$$

where the value of the dilution potential is in millivolts, and x is in equivalents of negative immobile charge per kilogram of water. Thus tissues of low colloidal charge density are characterized by dilution potentials that approach —12 mv. as a limit; this value represents the liquid junction potential between Solutions I and II. Such tissues may be either highly hydrated (relaxed pubic symphysis) or dense tissues in which the state of the colloid approximates electrical neutrality (Tendon, Table 1).

Following equilibration of the tissue with Solution III, the modified dilution potential, E_d', was found to be considerably lower, an effect which is attributed to combination of calcium ions with some of the immobile anionic groups. Accordingly,

$$E_d' = -12.3 + 215x' \tag{4}$$

The value —12.3 mv. represents the junction potential between Solutions II and III, and x' represents the modified density of colloidal charge following equilibration with Solution III. The difference $(x - x')$ is a measure of the bound calcium. Since the bound calcium lowers the density of immobile charges, sodium and chloride ions become more equally distributed across the boundary, the Donnan ratio tending toward unity. The nature of the ionic equilibrium is shown graphically (Chart 1).

* References 1 and 3.

TABLE 2.—*Colloidal Charge Densities (Initial and Modified) and Equilibrium Constants*

Species	Tissues	Condition*	Initial Colloid Charge Density, x (Eq. per Kg. H_2O)	Modified Colloid Charge Density, x' (Eq. per Kg. H_2O)	Ratio, x'/x	Equilibrium Constant, K
Monkey	Skin	N	0.072	0.044	0.61	0.024
		P	0.065	0.038	0.59	0.018
	Sternum	N	0.165	0.091	0.55	0.020
		P	0.134	0.084	0.63	0.026
	Gingiva	N	0.095	0.057	0.60	0.021
		P	0.063	0.041	0.65	0.029
	Dentin	N	0.141	0.097	0.68	0.036
		P	0.122	0.082	0.67	0.032
Rabbit	Epiphysis	N	0.146	0.099	0.68	0.036
		P	0.116	0.072	0.62	0.024
	Skin	N	0.067	0.041	0.61	0.021
		P	0.052	0.032	0.61	0.020
Mean					0.62 ± 0.04	0.026 ± 0.006

* N denotes normal state; P, stimulation by parathyroid extract.

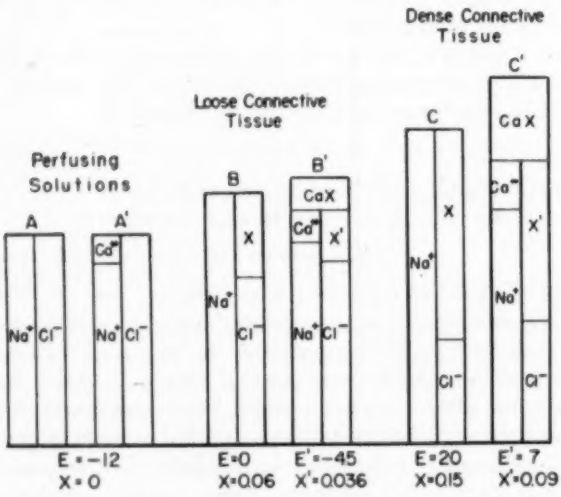

Chart 1.—Equilibrium distribution of ions between experimental perfusing solutions and connective tissues. A denotes isotonic NaCl solution, while A' denotes a solution of the same chloride concentration in which a small part of the sodium is replaced by calcium. B and B' denote states of loose connective tissue in equilibrium with A and A', respectively. In B', part of the total colloid, x, is combined with calcium to form an undissociated colloid, CaX. The remaining colloidal charge, x', determines a distribution of sodium, calcium, and chloride in accordance with the Gibbs-Donnan equilibrium. In B', the Donnan ratio tends toward unity. Thus the dilution potential, E_d', becomes negative (—4.5 mv.). C and C' illustrate related effects for dense connective tissue. In both B' and C', the value of x' is about six-tenths that of x. The change of the dilution potential is determined by $(x - x')$, and is —13 mv. for the dense connective tissue.

CALCULATION OF BOUND CALCIUM AND DETERMINATION OF A NOMOGRAM

By means of Equations 3 and 4 and the data of Table 1, the mean values of x and x' have been computed for the tissues studied in the normal state and following administration of parathyroid (Table 2). In addition, the ratio of x' to x has been computed for each of the tissues in the two states. The mean value of the ratio is 0.62, with a standard deviation of ± 0.04. Thus the calcium-binding properties of the six tissues, as reflected in the ratio, appear to be fairly constant in both normal and

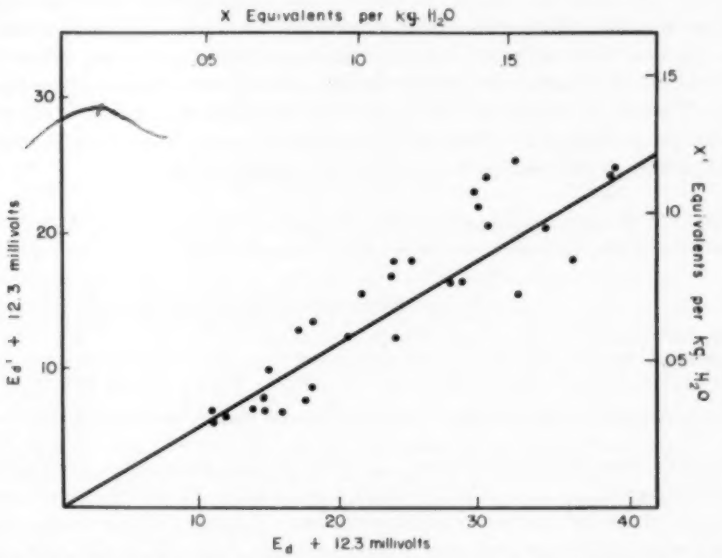

Chart 2.—Relation of modified dilution potential, E_d', to first dilution potential, E_d. The modified colloid charge density, x', is proportional to $(E_d' + 12.3)$. The initial charge density, x, is proportional to $(E_d + 12.3)$. The mean value of x'/x is 0.62, where x' and x are estimated in equivalents per kilogram of water.

parathyroid-stimulated states. The hormone decreases the value of x and of x', the ratio remaining constant. The data may be expressed (Chart 2) by the linear relation:

$$x' = 0.62x \tag{5}$$

To calculate the equilibrium constant of the reaction

$$Ca^{++} + X^{=} = CaX$$

the mass action law is applied:

$$\frac{(Ca^{++})^0 r^2 x'}{(x - x')} = K \tag{6}$$

where K is the equilibrium constant. $(Ca^{++})^0$ is the calcium ion molality of the applied solution (0.01 M). As an approximation, the Donnan ratio, r, is given by

$$r = 1 + (x'/0.3)$$

and

$$r^2 = 1 + 2(x'/0.3) + (x'/0.3)^2 \tag{7}$$

The numerator of Formula 6 represents the product of uncombined colloidal charge density and calcium ion molality in the tissue; the denominator represents combined calcium. Calcium ion is expressed as moles per kilogram of tissue water, while x' and bound calcium are expressed as equivalents per kilogram of water. A value of K is determined for each of the 12 states. The mean value is found to be 0.026 ± 0.006. Expressed exponentially, $K = 10^{-1.58}$.

The combination of calcium with serum proteins has been formulated by McLean and Hastings [12] as a similar bimolecular reaction. Their value for the constant K is $10^{-2.22}$. This indicates a considerably stronger affinity of calcium for blood proteins than for connective tissue colloids. At the same calcium ion concentration, blood proteins are therefore somewhat more highly saturated with calcium than are the connective tissue colloids. However, in dense connective tissues, where x' is high, the percentage saturation of the colloid increases greatly because of the Donnan effect. The sodium ion concentration of connective tissue is proportional to the colloidal charge density. Calcium ion concentration is proportional to the square, and bound calcium is proportional to the cube, of colloidal charge density.

TABLE 3.—*Comparison of Electrometric and Analytical Results*

	x (Eq. per Kg. H_2O)	r (Donnan Ratio)	$(Na^+)'$, mEq. per Kg. H_2O	$(Ca^{++})'$, mEq. per Kg. H_2O	CaX, mEq. per Kg. H_2O	Total Calcium, mEq. per Kg. H_2O	Equilibrium Constant K
Cartilage (electrometric)	0.165	1.70	255	7.2	29.7	36.9	0.020
Cartilage (direct analysis)*	.....	....	280.5	...	....	34.0	.....
Calculations from direct analysis..	0.200	1.87	(280.5)	9.7	25.3	(34.0)	0.034

* Results of Eichelberger and co-workers [2] converted to milliequivalents per kilogram of H_2O.

From the foregoing relations and any given value of E_d, the following variables may be estimated: x, x', r, (Ca^{++}), (Na^+), and (CaX). In addition, total calcium (exclusive of apatite or other crystalline deposits) may be obtained as the sum of bound calcium and free calcium ions. In order to check these values with independent values, they are compared with analytical figures for total calcium and sodium of cartilage (Table 3).

A calculation of the electrochemical state of osteochondral tissue is undertaken for monkey sternum in equilibrium with normal blood. The calculated data are compared with the analytical figures of Eichelberger, Brower, and Roma [2] for articular cartilage. From E_d, the dilution potential, 23.2 mv., the value of x is estimated as 0.165 Eq. per kilogram of tissue water. From this value, sodium ion concentration is estimated as 0.255 Eq. per kilogram of water.† Taking blood sodium as 0.15 M as an approximation, the Donnan ratio, r, is estimated as 1.7 and r^2 as 2.89. The estimated calcium ion concentration of the tissue is then 0.0072 Eq. per kilogram of water. This figure is based on a standard value of blood calcium ion, 0.0025 Eq. per liter.[12] From the tissue ionic calcium and the equilibrium constant K, taken as 0.020 (Table 2), the quantity of bound calcium, CaX, is estimated. The value is found to be 0.0297 Eq. per kilogram of water. The sum of ionic and bound

† Rather than the first approximation, a more exact quadratic equation is used to determine Na from x:

$$Na = \frac{x + \sqrt{x^2 + 0.09}}{2}$$

calcium is thus estimated as 0.0369 Eq. per kilogram of water. The analytical figure for total calcium is 0.0340 Eq. and that for sodium is 0.280 Eq., per kilogram of water.

The results of the two sets of observations thus appear to be in good agreement. This is noteworthy in view of the fact that cartilage also contains considerable amounts of magnesium and potassium. The former ion probably competes with calcium in combining directly with the colloid, an effect which should be considered in a more exact calculation. The results of the approximate calculation thus confirm the interpretation of the electrometric observations and indicate the validity of computations of electrochemical states from the data.

From the analytical figures, the converse calculation can be made, leading to an independent value of K, the mass action constant (Table 3). From sodium values for blood and cartilage, the Donnan ratio is determined. This value and the normal figure for blood ionic calcium yield the value 0.0087 Eq. for the ionic calcium of the matrix. Subtraction of ionic calcium from total calcium (analytical) yields the value of bound calcium. The value of x', the density of colloidal charge, is also obtained from $(Na^+)'$ or from r, the Donnan ratio. The values of x', ionic calcium, and bound calcium substituted in Formula 6 determine the value of K, the equilibrium constant, as 0.034, or $10^{-1.47}$. This agrees well with the value obtained electrometrically.

Further confirmation of the validity of the Gibbs-Donnan equilibrium in determining electrolyte distribution between blood and connective tissue is obtained by calculation of the product of sodium and chloride ion concentrations. Taking normal blood sodium as 150 mEq. per kilogram of water and chloride as 100 mEq., the product is 15,000 mEq.^{2} per kilogram.^{2} The ionic product for cartilage based on the analytical figures of Table 3 is 280×71, or 19,880 mEq.^{2} per kilogram.^{2} The result may be interpreted as indicating a lowering of the mean ionic activity coefficient of NaCl in ground substance by about 15%.‡ Part, if not all, of this effect is accounted for by the high ionic strength of cartilage, which is approximately twice that of blood (Donnan ratio about 1.9).

Calculations comparable to those of Table 3 have been made for other connective tissues and the results incorporated into a nomogram (Chart 3). In the computations the equilibrium constant has been taken as 0.026 to agree with the mean of six monkey and rabbit tissues (Table 2). Under endocrine stimulation, the density of colloidal charge may vary continuously over a wide range.§ Therefore x may be regarded as an independent variable determining the distribution of sodium, calcium, and other ions between blood and connective tissues. The nomogram illustrates the calculated distributions for the observed normal tissues and the extrapolated distribution for very low values of x (synovial fluid and other connective tissue fluids of low colloid content).[15] The distributions refer to normal blood electrolyte levels, with calcium ion and x taken as the two independent variables. Blood sodium is assumed constant at 0.15 M. In the monogram, the values of x refer to total negative colloidal groups, including those bound to calcium; the values of x' refer to free negative groups. The difference between x and x' represents equivalents of bound calcium.

‡ The mean ionic activity coefficient is defined as the geometrical mean of the ionic activity coefficients.[14]

§ References 1 and 3.

In the diagram, two limiting states are indicated; an upper limit of x, corresponding to about 0.2 Eq. per kilogram of water, and a lower limit of about zero. These two limits correspond, respectively, to dense tissues (cartilage, dentin, epiphysis, but not tendon), and to very soft tissues (highly relaxed pubic symphysis, synovial fluid, etc.). Both limiting states are coexistent with and in equilibrium with blood. With respect to water and electrolytes, all intermediate states of connective tissues are in equilibrium, the distribution being governed by the Gibbs-Donnan law and the calcium equilibrium constant. The nomogram is intended to apply only to tissues of approximately the same calcium-binding affinity. As such, it represents a homeostatic continuum in which the chemical potentials of sodium chloride, calcium chloride, and water are constant,‖ and equal to the chemical potentials of these components in blood or other perfusing fluids in equilibrium. The upper and lower

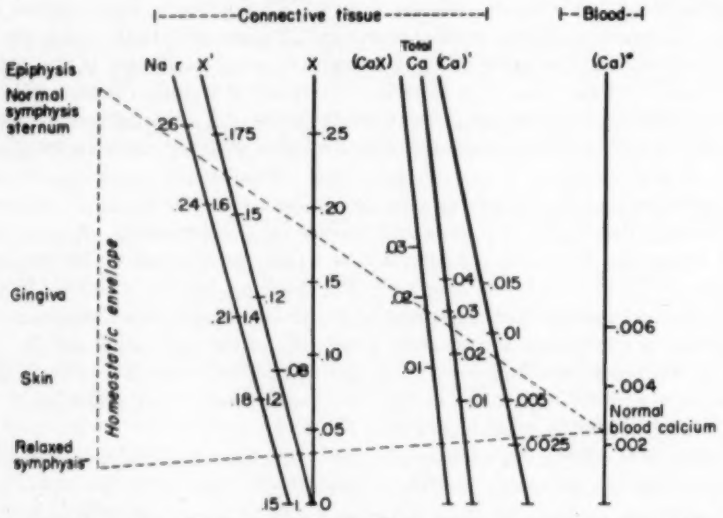

Chart 3.—Nomogram of sodium-calcium equilibrium between blood and connective tissues. Serum sodium is assumed constant at 0.15 Eq. per liter. Variables are tissue sodium, $^*Na$; Donnan ratio, r; equilibrium density of colloid charge, x'; total colloid charge density, x; bound calcium, CaX; total calcium; tissue ionic calcium, and serum ionic calcium. Excepting r, all values are in equivalents per kilogram of water. Bound calcium, CaX, represents the difference between x and x'. Varying states of connective tissue ranging from highly relaxed pubic symphysis to epiphysis are denoted. All states between the two limits are included. The homeostatic envelope encloses all intermediate states in equilibrium with normal blood sodium and calcium.

limits of the converging family of lines constitute a homeostatic envelope enclosing normal physiological states in equilibrium with normal blood sodium and calcium.

While the properties of this physiological continuum include constant chemical potentials of sodium and calcium salts, the concentrations of these components, as well as those of colloids, may vary over wide limits, and this is explicable on the basis of a heterogeneous equilibrium.[1] Connective tissue homeostasis is more clearly identified with constant chemical potentials than with salt concentrations.

‖ The Gibbs-Donnan equilibrium distribution depends on the assumption of constant chemical potentials.

HOMEOSTASIS IN HETEROGENEOUS SYSTEMS

In heterogeneous systems at equilibrium under Gibbs-Donnan conditions (Table 3) it is necessary to distinguish between the total quantity of a given component (sodium chloride or calcium chloride) in a certain mass or volume of the system and the quantities present in the different phases. If it is assumed that two coexistent submicroscopic phases are in equilibrium,[1] then for any substance (s) distributed between the two phases, a macroconcentration, C_s, and two microconcentrations, C_s' and C_s'', may be defined. The macroconcentration, C_s, is defined as the total mass of s per kilogram of water in the two-phase system as a whole. Each microconcentration, C_s' and C_s'', is defined as the mass of s per kilogram of water within each of the respective phases.

Then
$$C_s = C_s' M_w' + C_s'' M_w'' \tag{7}$$

and
$$M_w' + M_w'' = 1 \tag{8}$$

M_w' and M_w'' denote, respectively, the fractions of total water in Phases 1 and 2.

In particular, let C_s denote the density of colloidal charge, x, which is now assumed to be distributed between two submicroscopic coexistent phases in equilibrium. Then
$$x = M_w' z' + M_w'' z'' \tag{9}$$

where z' and z'' denote the densities of colloidal charge within the two phases. Each is expressed, like x, in equivalents per kilogram of water.

For the pubic symphysis, it has been shown that large changes of plasticity and hydration may occur at constant osmotic pressure and chemical potentials through a change of state involving the formation of water-rich Phase 1. Thus, if z' denotes the density of negative colloidal charge in the water-rich phase of ground substance, and z'' the density of colloidal charge in the colloid-rich phase, x may vary between z'' and z' as upper and lower limits. The upper limit of x has been found to approach 0.2 Eq. per kilogram of water (Table 2); lower limits of the order of 0.02 Eq. have been observed in synovial fluid.

On the nomogram, the upper and lower limits approximate, respectively, cartilage and synovial fluid. The equilibrium values for sodium, ionic calcium, bound calcium, and total calcium may be estimated for these two limits. Intermediate values of these variables between the two limits refer to macroconcentrations. The correlated value of x is also, according to Equation 9, a macroconcentration. It is theoretically possible, therefore, for x to vary, while z', z'', and the microconcentrations of bound calcium and the various ions remain constant within each phase. Physiological homeostasis may thus be referred to invariance of composition of individual phases and to constant chemical potentials of inorganic salts and water. Therefore macroconcentrations of electrolytes and colloids may vary over certain limits independently of microconcentrations. Cells surrounded by ground substance remain, under such conditions, in an invariant environment.

It is convenient to refer to two general sets of properties involved in any change of thermodynamic state. Any property of a simple solution, for example, which is proportional to the total quantity may be defined as an extensive variable. Included in this class are the volume, mass, entropy, free energy, etc. Those variables which

are independent of total quantity and which depend only on composition may be defined as intensive. They include concentrations, chemical potentials, osmotic pressure, and vapor pressure.

In terms of these definitions, the macroconcentrations of colloid and electrolytes in connective tissue may be understood normally as extensive variables which depend on the relative quantities of coexistent water-rich and colloid-rich phases occurring in a given part of the system. The microconcentrations within each phase are intensive properties, as are the chemical potentials of water and electrolytes. Thus, homeostasis depends essentially on the maintenance of invariant intensive properties. Functional adaptations, such as the relaxation of the pubic symphysis, responses of

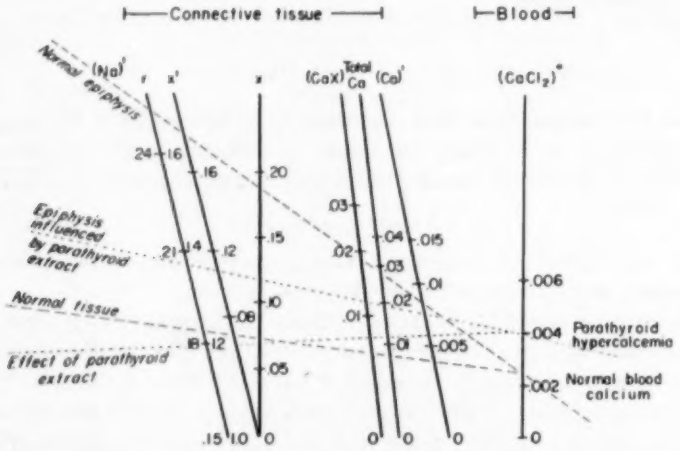

Chart 4.—Nomogram of sodium-calcium equilibrium as affected by parathyroid extract. Significance of symbols is same as in Chart 3. Effect of hormone on epiphysis is to decrease colloidal charge density, x, resulting in lowered calcium content. The modified state is determined by lowered value of x and increased level of serum calcium Equilibrium of other tissues is determined by elevated, nonhomeostatic serum calcium.

the epiphysis to parathyroid stimulation, and hydration of sex skin in response to estrogens, may thus be considered homeostatic as long as changes of blood electrolyte concentrations are not induced. One set of properties, the extensive, is thus adaptable, and may vary, while the intensive properties remain constant.

NONHOMEOSTATIC EFFECTS OF PARATHYROID EXTRACT

When an animal is treated with large amounts of parathyroid, there is a disaggregation of the connective tissue matrix of bone, cartilage, and some soft tissues,[6] leading to a fall in colloidal charge density in these regions. Simultaneously, blood calcium rises, leading to the establishment of new and nonhomeostatic relationships between the variables. A nomogram (Chart 4) illustrates the nature of these changes. The dotted lines represent the envelope of modified states of dense and loose connective tissues, from which values of the variables may be read. A noteworthy situation arises in that values for ionic, bound, and total calcium of soft connective tissues may actually rise, despite a considerable fall of these values in dense connective tissues, such as bone and cartilage.

CALCIFICATION IN CONNECTIVE TISSUES

The calcium content of tissues, exclusive of any apatite phase which may be present, is related to the density of the negatively charged colloid of the tissue. The highest values for ionic and bound calcium are therefore to be found in dense connective tissues. Since hormones act on connective tissues in such a way as to vary the state of aggregation of the ground substance, it is to be expected that these will, under certain conditions, affect the distribution of calcium in the same way as they alter the concentration of other electrolytes and of water. Calcium concentration is determined in part by the Donnan distribution, and in part by chemical binding. The latter is illustrated by the results of Neuman and co-workers,[16] showing a high affinity between calcium and chondroitin sulfate, an important fraction of the connective tissue colloids. Accordingly, some dense tissues, such as cartilage, contain ionic calcium at concentrations as high as three times that of blood, while bound calcium concentrations may exceed blood values by a factor of 10. Ionic calcium is proportional to the square, and bound calcium is proportional to the cube, of the colloidal charge density. A comparison of blood, skin, and sternum has been made (Table 4). In the absence of apatite formation, by present convention, tissues containing large quantities of bound calcium would be termed "uncalcified."

TABLE 4.—*Calcium Concentrations of Blood and Tissues*

	Ionic Calcium $(Ca^{++})'$, mEq. per Kg. Water	Bound Calcium (CaX), mEq. per Kg. Water	Total Calcium, mEq. per Kg. Water
Skin	3.7	4.8	8.5
Sternum	7.2	29.7	36.9*
Blood [18]	2.5	2.5	5.0

* Exclusive of apatite.

In histochemical studies on the glycoprotein matrices of tissues undergoing calcification, an altered staining reaction with the periodic acid-Schiff reagent has been observed. An increased intensity in coloration or reactivity is thus characteristic of sites of pathologic calcification, of newly calcifying bone, and of epiphyseal cartilage matrix contiguous to the zone of ossification.¶ It has been proposed that in the latter instance the ground substances is depolymerized as a preliminary to calcification.#

This hypothesis may be related to the present work in the following way: A lowering of the density of the colloid of cartilage releases calcium ions locally. Concurrent elevation of phosphate ion levels in these areas provides conditions favorable to the deposition of calcium phosphate. Similarly, a local metabolic increase in the concentration of bicarbonate ions should favor the formation of a crystalline phase. Other bivalent cations, such as magnesium, also react with connective tissue colloids. These reactions may be competitive, with each complex characterized by its free energy of formation and by its dissociation constant. Such considerations could explain the results of Sobel * with cartilage slices immersed successively in solutions of different cations.

¶ References 17 to 19.

References 17 and 18.

* References 20 and 21.

Complexes with calcium are formed by many substances present, or potentially present, in tissues, such as blood and tissue proteins, amino acids, and dicarboxylic and tricarboxylic acids.† Neuberg and Mandl [23] and Mandl and co-workers [24] list a series of biological substances affecting the solubility of calcium salts. In particular, the concentration of citrate as affected by cellular metabolism may be expected to enter into equilibriums involving calcium. Calcium may thus be considered to be partitioned between the colloids of ground substance and a number of other tissue components. Any change of state of ground substance induced by hormonal stimulation must then lead to a redistribution of calcium among the various components, as well as to a change of electrolyte equilibrium with blood.

SUMMARY

An electrometric method has been applied to the estimation of the calcium-binding properties of various connective tissues. Displacement of the boundary potential by 0.1 isotonic saline was determined before and after exposure of the junction to 0.01 M calcium chloride rendered isotonic with sodium chloride, and the difference between these values is considered to be a measure of calcium bound by immobile, negatively charged colloid of the tissue. Tissues studied included tibial epiphysis, abdominal skin, and gastrocnemius tendon, in the rabbit and osteochondral areas in the sternum, dentin, gingiva, and skin, in the monkey. These tissues were also studied in animals receiving parathyroid extract. In all tissues studied, a relatively constant fraction of connective tissue colloid was bound by calcium. The equilibrium constant for combination of calcium with connective tissue colloid was $K = 10^{-1.58}$.

From the foregoing relations, the following variables may be computed in any given instance: colloidal charge density, colloidal charge density modified by calcium, the Donnan ratio, tissue calcium ion and sodium ion, bound calcium, and total calcium. For monkey sternum the calculated values of these quantities agree well with the analytical figures of Eichelberger, Brower, and Roma for articular cartilage. The Gibbs-Donnan equilibrium may therefore be used to determine electrolyte distribution between blood and connective tissue. A nomogram exhibiting the above variables for several connective tissues in equilibrium with blood is presented. Calcium ion concentration is proportional to the square, and bound calcium to the cube, of colloidal charge density, while sodium varies linearly.

Practical and theoretical topics arising from these relationships are discussed, namely, concentrations of ionic and bound calcium in dense connective tissue (especially cartilage) and its relation to calcification, modification of calcium distribution by the action of hormones on connective tissue, nonhomeostatic effects of parathyroid hormone, pathological calcification, and role of other calcium-binding constituents of tissues in modifying calcium distribution.

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HOMEOSTASIS OF CONNECTIVE TISSUES

II. Potassium-Sodium Equilibrium

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CHICAGO

IN THE responses of organs and tissues to hormonal action there is abundant evidence of connective tissue participation.* Where the hormonal action seems to be directed toward the connective tissue itself, as with cortisone and relaxin, the lability of the tissue may become particularly striking. In either case the ensuing changes in state of the connective tissue are associated with shifts in the distribution of colloid, water, and electrolytes, which may be characterized in electrochemical terms.† For the pubic symphysis of the guinea pig, it was shown that hormones may effect wide variations in size, plasticity, and water content at constant osmotic pressure, provided that the behavior of the tissue is that of a two-phase system at equilibrium. In such a case, conditions of thermodynamic equilibrium may permit changes in colloid, water, and electrolytes consonant with maintenance of physiological homeostasis.

For blood, the dependence of physiological homeostasis on thermodynamic equilibrium was the subject of Henderson's exhaustive investigation.[8] Treating blood as a two-phase system, and reasoning from Gibbs's theoretical principles,[9] he expressed in terms of d'Ocagne nomograms the large number of variables necessary to characterize the system. Equilibrium states involving all the variables may be expressed in relation to any two arbitrarily chosen independent variables. By these means, an exact understanding of the physicochemical state of a biological system as a whole became possible. The general method led to a detailed description of the maintenance of a constant milieu interne under conditions of physiological functioning, and the biological principle of Claude Bernard [10] was thus related to the laws of thermodynamics. This principle was applied also by Cannon in studies of organization for physiological homeostasis.[11] More recently, Selye has utilized the idea of displacement of homeostasis as a "stress" leading to adaptations mediated through endocrine systems.[12]

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* References 1 to 5.

† References 6 and 7.

A correspondence between conditions for physiological homeostasis and thermodynamic equilibrium may be postulated throughout the organism.[5] In connective tissues the large number of variables characteristic of any state are related by equilibrium conditions to a smaller number of independent variables. A description of equilibrium states, as for blood, should contribute to the correlation of biological behavior with thermodynamic state. By electrometric methods, a nomogram of the equilibrium of sodium and calcium with connective tissues has been worked out and compared with analytical results.‡

A more complete description of the interaction of physiologically important electrolytes with connective tissues requires data on potassium ions. It has been found in the pubic symphysis of the female guinea pig that the mobility of potassium ion depends on the density of immobile negatively charged colloid of the tissue.[6] The relative ionic mobility was found to be low in the unrelaxed symphysis and high in the hydrated state characteristic of relaxation. The results suggested that the tissue behaves as a cation exchange resin, concentrating potassium in the unrelaxed state but behaving nonselectively in the relaxed state. Potassium appeared to be the only physiological ion to be selectively immobilized in this manner. Calcium has been shown to form undissociated complexes with the colloid, also tending to displace sodium. On the basis of these results, the electrolyte content of connective tissues appears to depend on the nature and quantity of the colloids and on equilibrium constants characteristic of the ions.

In the present study, electrometric determinations and histochemical observations were performed on the same tissues, before and after the use of hormones to effect changes in state of the tissues. Electrometric studies yield data on the density of negatively charged, immobile colloid and on the relative mobilities of ions,[6] while histochemical studies give information on the nature and state of aggregation of colloids and on their solubilities.§ Methods previously applied to the pubic symphysis in normal and relaxed states have been extended to several other types of connective tissue, and the thesis of connective tissue as a two-phase continuum [6] has been further developed. In particular, the interaction of potassium and sodium with tissues has been studied and compared with that of calcium and sodium.

ELECTROMETRIC METHODS AND MATERIALS

Animals were anesthesized with pentobarbital sodium or ether or both. The reference junction with isotonic saline was formed by the insertion of a 20-guage needle under the skin of the abdomen of the animals, and under the skin of the shoulder of the birds. For the experimental junction, a similar needle cut to 1.5 cm. length was used. It was inserted in the tissue under investigation as follows: skin, sex skin, sternum, and gingiva of the monkey; skin of rat; skin and comb of capon; pubic symphysis of the guinea pig. Details of the circuit used have been described.‖ In the present series, NaCl dilution potentials and potassium substitution potentials were determined. The former is the displacement of potential obtained when a 1/10 dilution of isotonic NaCl is substituted for isotonic (0.15 M) NaCl at the experimental junction. The potassium substitution potential is the displacement obtained when isotonic (0.15 M) KCl is substituted for isotonic NaCl. In general, steady readings were obtained within

‡ References 13 and 14.

§ References 2, 15, and 16.

‖ References 6 and 7.

10 to 30 seconds after replacement of one electrolyte solution by another. Duplicates generally agreed after two or three flushings. Hereafter the NaCl dilution potential will be denoted as E_{Na}, and the potassium substitution potential, as E_K. A summary (Table 1) is presented, giving the animals and tissues studied with the respective hormones and doses employed.

Monkeys.—Prepuberal females were used. The sex skin appeared bilaterally as a translucent oval area. The animals were anesthetized and determinations made on the several areas; immediately after this biopsies were made of sex skin and gingiva. One monkey received 500 R. U. of pregnant mare serum gonadotropin subcutaneously, followed by estrogen intramuscularly. The other six animals received, intramuscularly, 1 mg. of estradiol benzoate (6,000 R. U.) in oil, in two doses 24 hours apart. A very pronounced sex skin response, involving the pubic and circumanal region, appeared in the course of the next few days. Animals were studied electrometrically on the fifth or sixth day following the first injection, when the reaction was judged to be maximal, and occasionally a week later, when the reaction had subsided. After readings at maximal reaction, biopsy specimens of sex skin and gingiva were again taken at sites removed from the previous ones. Pubic and anal regions of swelling did not differ in their electrometric responses.

TABLE 1.—*Summary of Tissues, Hormones, and Dosages*

Animal	Condition	Number	Hormone	Dose	Tissues
Monkey, Macacus mulattas	Immature females	7	Mare serum gonadotropin Estradiol benzoate	500 R. U. 0.5 mg./day for 2 days	Skin, sex skin, gingiva, sternum
Rat	Young adult females	8 8	Cortisone Untreated	5 mg./day for 1-21 days	Skin
Capon	Young adult	4	Testosterone proprionate	1-5 mg./day for 10-20 days	Skin, comb
Guinea pig*	Young adult females, normal and castrated	17	Estrogen	1.25 γ/day for 4 days 1/8-32 palpation units (single dose)	Pubic symphysis
	Pregnant 2-40 days prepartum	25	None	.....	Pubic symphysis
	Postpartum	16	None	.....	Pubic symphysis

* Joseph, Engel, and Catchpole [6]; Catchpole, Joseph, and Engel.[7]

Rats.—Young adult females were used. One group was given cortisone, 5 mg. per day for 1 to 21 days, and studied at intervals during this period, along with a group of pair-fed controls. Animals were anesthesized with ether, and potentials were determined on skin of the right flank Skin biopsies from the interscapular area were made.

Capons.—The first two birds used may not have been completely caponized; however, this would not seem to affect the interpretation of the experiment. Initially their combs were small and pale, and following administration of 1 mg. per day of testosterone propionate in oil intramuscularly for 10 days a considerable increase in size was obtained. Needles were inserted deep into the substance of the comb near the base of one of the points. Biopsy specimens, obtained before and after treatment, were secured by making a wedge-shaped incision in the comb at the base of a point. The point so obtained was bisected longitudinally before freezing. Healing was rapid. These animals were observed later to have developed rather considerable appendages, indicating that testicular remnants may have been present. Two other birds, apparently completely caponized, were given 5 mg. of testosterone propionate daily. Combs grew vigorously and were studied as above. Skin of the shoulder region was also studied.

Guinea Pigs.—Pentobarbital and ether anesthesia was used, and the technique and materials of symphyseal potential measurements have been described elsewhere.[6] Relaxation was induced by administration of relaxin following estrogen priming.

RESULTS

Electrometric.—Data for the present series of tissues have been assembled (Table 2; Fig. 1) to indicate the dilution and substitution potentials found in normal (rats, monkeys) or castrate (capons) tissues before and after modification by hormonal treatment. Also indicated in Table 2 are results obtained previously for the highly variable connective tissue of the pubic symphysis of guinea pigs. It is evident that normal connective tissues form a progressive series, varying from the dense tissue of sternum or unrelaxed symphysis, with dilution potentials of 20 to 30 mv., to the loose tissue of relaxed symphysis, with dilution potentials of —6 to —10 mv. Between these limits, other tissues find characteristic positions, for example, normal monkey gingiva, monkey skin, capon comb, monkey sex skin, in descending order of colloidal density. As previously described,[6] for each electro-

TABLE 2.—*Effects of Hormones on Electrochemical State of Tissue*

Species	Tissue	Hormone	Dilution* Potential E_d, Mv.		Potassium* Substitution Potential E_K, Mv.		Density of Colloidal Charge X,† Eq. per Kg. H_2O	
			Untreated	Treated	Untreated	Treated	Untreated	Treated
Monkey	Sex skin	Estrogen	+0.6 ±2.2	—7.0 ±1.4	—0.1 ±1.2	—1.4 ±1.6	0.000	0.025
	Skin	Estrogen	+4.1 ±1.2	+0.6 ±1.9	+1.2 ±0.6	—0.2 ±0.6	0.070	0.060
	Gingiva	Estrogen	+8.1 ±1.5	+2.6 ±1.1	+2.4 ±0.9	+0.7 ±0.6	0.095	0.069
	Sternum	Estrogen	+21.2 ±3.1	+14.8 ±2.1	+6.9 ±1.1	+4.1 ±1.1	0.156	0.126
Capon	Comb	Testosterone	+2.0 ±1.3	—8.2 ±3.0	+0.5 ±0.8	—2.8 ±1.0	0.006	0.019
	Skin	Testosterone	+4.7 ±1.7	+4.2 ±2.1	+1.4 ±0.5	+1.1 ±0.7	0.079	0.077
Rat	Skin	Cortisone	+3.6 ±1.5	—4.0 ±1.9	+0.8 ±0.8	—1.5 ±0.8	0.074	0.069
Guinea pig‡	Pubic symphysis	Relaxin (after estrogen priming)	+24.0 ±6.2	—0.2 ±2.9	+5.4 ±1.9	—1.0 ±1.1	0.170	0.056

* Mean and standard deviation.
† Mean values from E_d and Equation 1.
‡ Values from Joseph, Engel, and Catchpole.[6]

chemical state, a value, x, can be computed, which is the apparent density of negative colloidal charge of the tissue, and which is correlated with the potassium substitution potential and potassium mobility. After treatment with estrogen, androgen, and cortisone in the several species, there occurs in general a fall in the value of x. This has been interpreted as due to formation of soluble colloids of the ground substance, leading to uptake of water and electrolytes from the blood. Histochemical correlates of these electrochemical changes will be presented in the next section, and further theoretical implications, in a later section. It is noteworthy that estrogen, while displacing downward the dilution potentials for all tissues, does not in the monkey disturb the order of the sequence: sternum, gingiva, abdominal skin, sex skin. After hormonal treatment, it is interesting to note that the abdominal skin, for example, shows the same potential as untreated sex skin. It was also observed that both normally and under the influence of hormones the various types of tissue were isopotential. Thus in the monkey, sex skin, abdominal

skin, gingiva, and sternum showed only very small potential differences when any two isotonic NaCl junctions were compared. In the other species, also, the resting potentials were typically very small, indicating in general that the various types of connective tissue are normally at the same potential.

Histochemical Methods.—Small pieces of a number of tisuses, including monkey sex skin and gingiva, rat skin, and capon comb, were excised for histochemical study before and after hormone stimulation. These were immediately frozen in isopentane chilled in liquid nitrogen (— 140 to — 160 C.), and subsequently dehydrated in vacuo at about — 32 C. The frozen-dried tissues were infiltrated and embedded in paraffin (m. p., 60 to 62 C.). Sections were cut at thicknesses of 4 to 20 μ. These were mounted on albuminized slides with use of finger pressure after slight warming over a hot plate. The slides were deparaffinized in petroleum benzin (reagent) before further treatment with reagents.

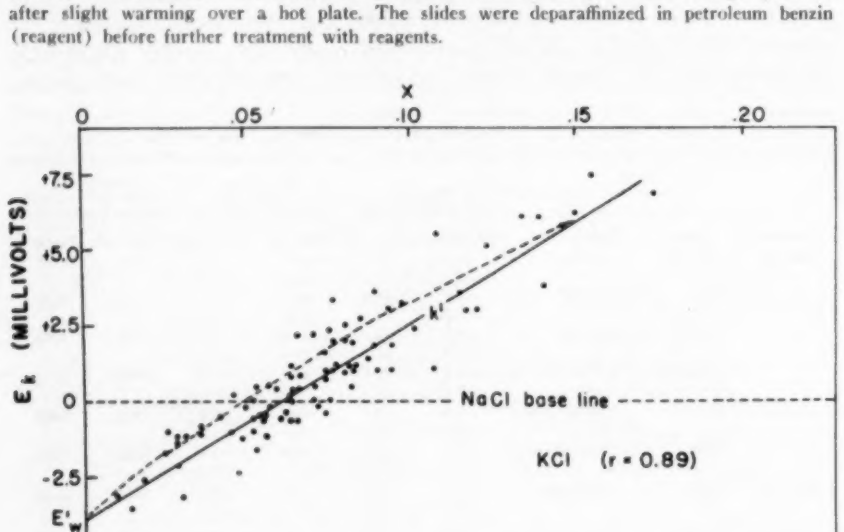


Fig. 1.—Correlation of potassium substitution potential (E_K) with NaCl dilution potential (E_d) for monkey, rat, and capon tissues. Solid curve: Equation 2 with slope k' and intercept E_w'. Dotted curve: potassium mobilities from Equation 3, substituted into Henderson equation.[6]

The carbohydrate-protein complexes were identified by the periodic acid-leucofuchsin method of McManus,[17] Lillie,[18] and Hotchkiss,[19] as described by the last author. This involves the oxidation by the periodic acid of hydroxyl groups in the 2 and 3 position of the saccharide. The polyaldehydes thus formed develop a red insoluble compound with the Schiff reagent. While substances other than carbohydrates may give this reaction, its specificity can be reasonably well established by pretreatment of the tissue with reagents and enzymes.[15] This also helps to characterize further the carbohydrate-containing substance. Soluble carbohydrates can be removed by pretreatment with water; glycolipids can be eliminated by overnight extraction in hot methanol-chloroform; glycogen is digested by β-amylase.

The histochemical part of this investigation was principally concerned with the demonstration of a water-soluble carbohydrate-containing fraction, as well as the insoluble residue. (In the theoretical part these fractions are substantially identified with the two phases of a heterogeneous system.) For this purpose, the sections were treated with several drops of M/15 phosphate buffer (pH 7) for one hour. They were then rinsed with buffer and denatured overnight in

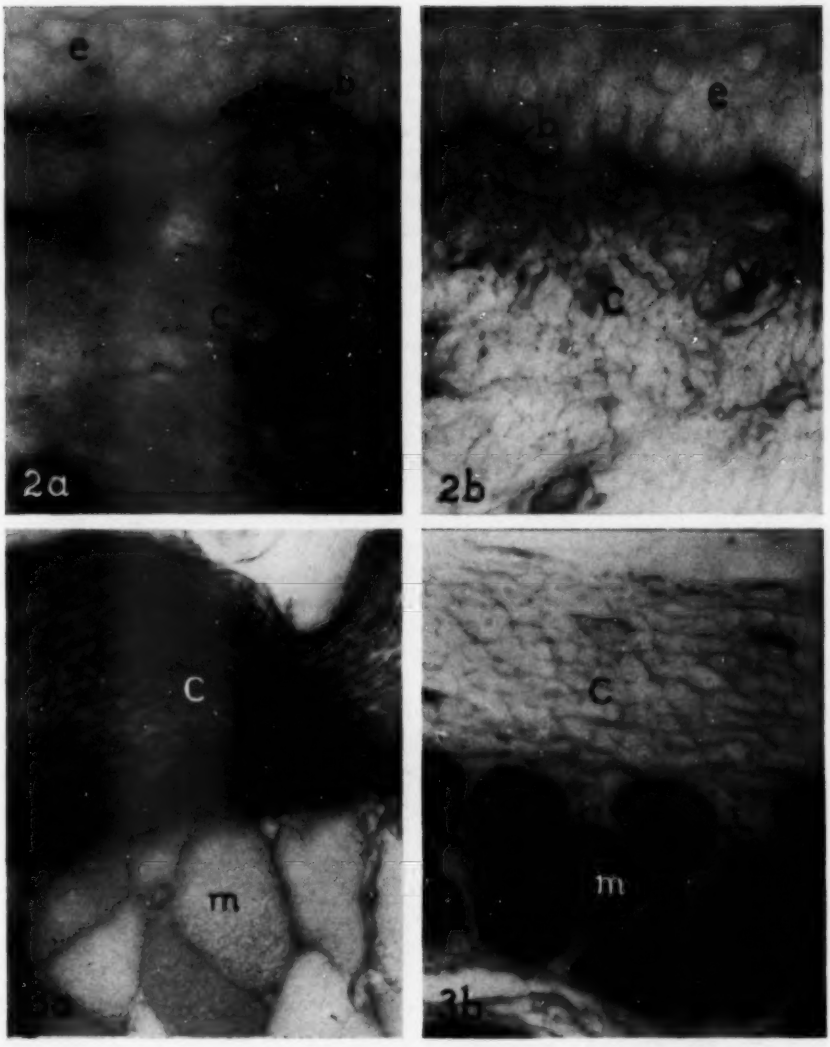
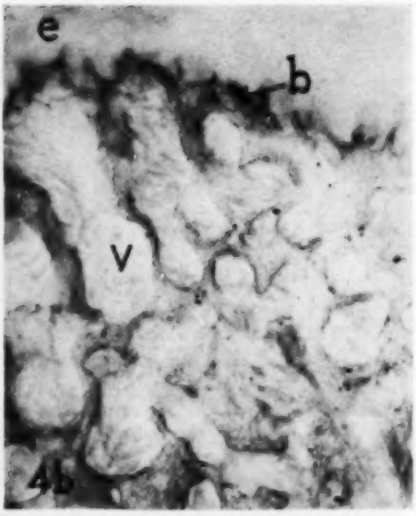
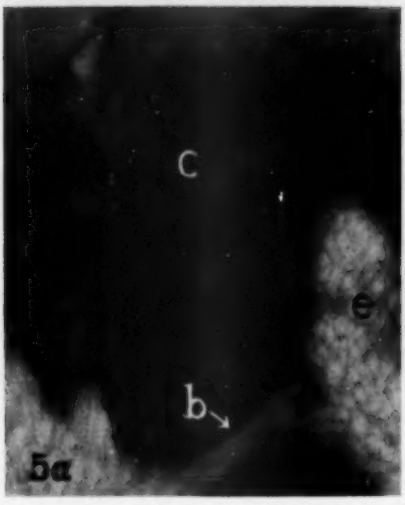


Fig. 2.—In this Figure and in Figures 3 to 5, tissues were fixed by freezing-drying and embedded in paraffin. Sections were cut at 6μ except in the case of Figure 3, and the paraffin was removed in petroleum benzin (reagent). After denaturation in 95% ethanol, the glycoprotein ground substance was demonstrated with the periodic acid-leucofuchsin method of Hotchkiss.[17] *b*, basement membrane; *c*, connective tissue; *e*, epihelium; *m*, muscle; *v*, blood vessel; *x*, equivalents per kilogram of connective tissue water. (*a*) Sex skin of prepuberal female monkey. The connective tissue ground substance appears fairly homogeneous, and the subepithelial and perivascular basement membranes are heavily defined. $x = 0.060$. $\times$ 770. (*b*) Sex skin of prepuberal female monkey following stimulation with 6,000 R. U. of estrogen. The ground substance is less densely stained, and all basement membranes appear attenuated. $x = 0.025$. $\times$ 770.

Fig. 3.— (*a*) Intrascapular skin of rat. The connective tissue of the hypodermis is compact and stains deeply, while that between muscle bundles stains lightly. $x = 0.074$. $\times$ 770. (*b*) Interscapular skin of rat following administration of 75 mg. of cortisone over period of 15 days. The connective tissue of the hypodermis resembles a loose network, and ice crystal artifacts are prominent. The muscle fibers are separated, and the carbohydrate-containing proteins of the muscle are now deeply stained. $x = 0.039$. Section cut at 20μ. $\times$ 770.





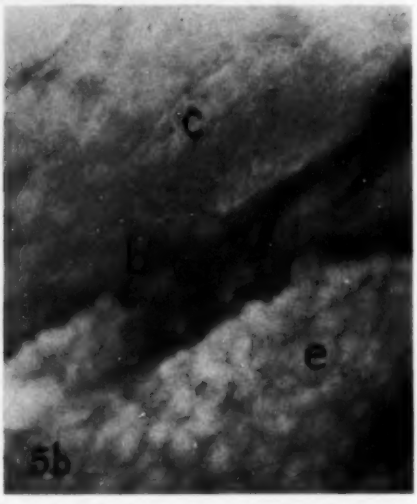


Fig. 4.—(*a*) Comb of capon. The dense fibrous subepithelial zone is embedded in a deeply stained ground substance. $x = 0.066$. × 770. (*b*) Comb of capon following administration of 10 mg. of testosterone propionate over a three-week period. The fibrous architecture is considerably modified, and now loosened; the ground substance is attenuated. $x = 0.019$. × 770.

Fig. 5.—(*a*) Gingiva of immature female monkey. The ground substance appears optically homogeneous. The subepithelial basement membrane is strongly defined. $x = 0.095$. × 770. (*b*) Gingiva of immature female monkey following injection of 6,000 R. U. of estrogen. The ground substance is now more weakly stained, and basement membranes are less prominent. $x = 0.069$. × 770.

95% ethanol. Subsequently they were stained, and the diminution in the stainability was regarded as evidence for the solubility of carbohydrate-containing material in the connective tissue ground substance. In an attempt to identify hyaluronic acid and chondroitin sulfuric acid moieties in the ground substance, some of the preparations were also exposed for two hours to testicular hyaluronidase (250 viscosity-reducing units per cubic centimeter) in *M/15* phosphate buffer (pH 7) or M/10 acetate buffer (pH 4.5). Certain details of morphology were studied in sections counterstained with methyl green (chloroform-extracted) and in sections stained with hematoxylin and eosin.

Histochemical Results.—Detailed histological descriptions of the several tissues are not included, since they essentially confirm the studies of others.¶

Monkey Sex Skin Following Estrogen.—In the unstimulated state this skin is but slightly differentiated by a slight degree of swelling. In the sections, the ground substance appeared almost homogeneous. The subepithelial basement membrane was a well-defined dark-red linear structure. Blood vessels were clearly defined by their prominent basement membranes. The connective tissue cells were relatively small, and in some the cytoplasm stained pale pink.

After the injections of estrogen, the sexual areas swelled and reddened. The ground substance was then more lightly stained with the periodic acid-leucofuchsin reagent, and the appearance of homogeneity in the connective tissue gave way to a loose fibrillar arrangement. The subepithelial and perivascular basement membranes became much thinner. Ground substance could be dissolved from sections of both resting and stimulated sex skin, as shown by decreased stainability following buffer extraction. However, the swollen sex skin showed a greater decrement in staining, suggesting the presence of more water-soluble glycoprotein. This stimulated state was characterized by the appearance of large, elongated connective tissue cells, the cytoplasm of which contained optically homogeneous or granular material, apparently glycoprotein in nature.

Monkey Gingiva Following Estrogen.—The attached gingiva in the area of the deciduous canine diastema was studied. This is a firm pink tissue, adherent to the underlying alveolar bone. The connective tissue layer had a more compact fibrous quality than sex skin, and the ground substance stained more heavily. The subepithelial basement membrane was clearly outlined. Following treatment with estrogen, the gross appearance of the gingiva remained essentially unchanged. In those animals showing the greatest electrometric changes, where the dilution potentials decreased from about 10 mv. to about 2 mv., the ground substance was clearly altered. This was shown by a lighter staining and subtle thinning of the subepithelial basement membrane. Water extraction dissolved somewhat more ground substance from the stimulated gingiva. With smaller changes of potential, the differences between stimulated and unstimulated states were not clearly defined. Differences in intracellular glycoproteins could not be established. In both states some fibroblasts contained very small granules of cytoplasmic glycoproteins.

Capon Comb as Influenced by Testosterone.—Individual processes ("points") of the comb were amputated for examination. In the quiescent state, each point had a thin fibrous core which was associated with a deeply stained ground substance. The intermediate and subepithelial connective tissue layers were progressively somewhat less dense. The epithelium was demarcated from the connective

¶ References 2, 5, and 20 to 25.

tissue by a prominent basement membrane. The entire structure was moderately vascularized, and the basement membranes of the blood vessels were prominent. After multiple injections with testosterone, the comb enlarged to several times its original size; it was reddened and had increased turgor. On histochemical examination, the ground substance appeared greatly attenuated in its distribution, and there were now many vascular sinuses throughout the connective tissue. The fibrous architecture had been converted to a much looser structure; this change was most advanced in the intermediate zone. Here the connective tissue cells were larger than in the untreated tissue. They reacted only lightly with dyes and stained pale pink with the periodic acid-leucofuchsin reagent. Treatment of sections of both resting and stimulated comb with buffer removed considerable quantities of carbohydrate-containing material. Ice-crystal artifacts, characteristic of tissues with high water content, were a prominent feature of the stimulated comb.

Rat Skin Under the Influence of Cortisone.—In the normal skin, the superficial part of the dermis as stained with the periodic acid-Schiff reagents appeared fairly homogeneous. It was demarcated from the epidermis by a delicate basement membrane. The deeper layers of the dermis and the hypodermis contained concentrations of fat cells and were penetrated by striated muscle, the fibers of which were enveloped by interstitial connective tissue. The hypodermis was less dense, the collagen fibers more apparent, and, on the whole, the concentration of ground substance appeared less than that observed in the more superficial connective tissue layer.

After treatment with cortisone, even tactile examination showed the skin thickness to be reduced. This was due largely to a reduction in the thickness of the adipose subcutaneous connective tissue layer. The ground substance of the papillary layer did not appear greatly altered. In several animals, including two treated for 20 days, a delicate thinning of the subepithelial basement membrane could be noted. In the hypodermis a more pronounced effect was observed. The tissue had a loose fibrillar arrangement, and the stainability was reduced. Ice crystals were prominent, indicating water uptake. The muscle bundles of the hypodermis were often separated. This appeared to reflect a state of edema, or of solution of a part of the interstitial connective tissue. The staining of the muscle bundles was also altered; while formerly they were generally pale, after treatment with cortisone, they became intensely stained. Since this increased stainability was only partially reduced by buffer or β-amylase, some change in a protein component of the muscle is suggested. Differences in the water solubilities of ground substance in sections from treated and untreated animals could not be clearly established. The connective tissue cells in both groups contained fine cytoplasmic granules identified as glycoprotein.

Hyaluronic acid and chondroitin sulfuric acid have been previously demonstrated in some of the tissues used in this investigation.# Our attempt to localize these substances by histochemical methods led to inconsistent results. This may be due in part to their ready solubility in the buffer alone or, in the case of hyaluronic acid, to a failure to react with the periodic acid-leucofuchsin reagent.

As shown in the previous section, hormonal treatment leads, in the various tissues, to a shift in the NaCl dilution potentials toward negative values, interpreted as a decrease in the density of immobile, negatively charged colloid. Potential measurements thus reflect a change in colloidal aggregates generally. On the other

References 22, 26, and 27.

hand, the histochemical studies refer to the behavior of carbohydrate-containing colloidal entities only. Nevertheless, it would appear that there is a considerable area of agreement between the two sets of observations. Previous work has shown that disaggregation of glycoproteins gives rise to characteristic changes in morphology, stainability, and solubility of connective tissue components.* On the basis of these criteria of change, all tissues studied gave evidence of glycoprotein involvement in the response. Thus, after treatment with the several hormones, sex skin, gingiva, comb, dermis, and hypodermis showed a paler staining with the leucofuchsin reagent. Sex skin and gingiva showed thinning of basement membranes and increased water solubility of glycoprotein, while comb and hypodermis showed a looser fibrillar pattern. Ice-crystal artifacts, indicative of increased water content, were prominent in comb and hypodermis. Previous work with the pubic symphysis showed decreased stainability, thinning of basement membranes, increased solubility of the ground substance, and increased in vivo uptake of Evans blue during relaxation. It should be noted that the apparent degree of participation in these responses may depend on the initial state of the tissue; for example, unstimulated comb already shows considerable amounts of water-soluble glycoprotein. The relative amounts of glycoprotein and other negatively charged colloids may also modify the response. The reactions cited are consistent with glycoprotein disaggregation as an important feature of the action of the hormones studied on tissues. The formation of soluble fractions of colloid, compensated by changes in electrolyte distribution and uptake of water, gives a basis for interpretation of the electrometric findings.

RELATION OF JUNCTION POTENTIALS TO STATE OF CONNECTIVE TISSUE

In the treatment of experimental results, the electrochemical principles used in studies of the pubic symphysis † have been applied. An empirical relation between potassium mobility and the electrochemical state of the tissue was derived from the potassium substitution potential. Statistical summaries of the electrometric data, including NaCl dilution potentials and KCl substitution potentials, are presented (Table 2). It has been shown that the dilution potential, E_d, depends on x, the immobile negative charge density, according to a linear approximation formula,

$$E_d = -12.3 + 215x \qquad (1)$$

where the constant —12.3 mv. is the value of the liquid junction potential between 0.15 M and 0.015 M NaCl solutions. The colloidal charge density is expressed as equivalents per kilogram of water.

It has also been found that the potassium substitution potential, E_K, is correlated with x by a linear formula,

$$E_K = E_w' + k'x \qquad (2)$$

where the intercept, E_w', is the value of E_K extrapolated to zero value of x and k' is the slope. Equation 2 has been derived by application of Henderson's theory of liquid junction potentials.‡ Values of E_w' and k' have been computed from the data by the method of least squares (Fig. 1; Table 3).

* References 2, 16, and 28.

† References 6 and 7.

‡ References 6, 29, and 30.

Also, by means of the Henderson theory, a relation between the relative potassium mobility, u_K, and x has been deduced [6]:

$$u_K = u_K^0 - c\sqrt{x} \qquad (3)$$

where u_K^0 is the value of u_K extrapolated to zero value of x and c is a constant. Equation 3 is of the same form as that derived by Onsager for ionic mobilities in simple salt solutions.[21] Values of u_K^0 and c have been derived from the data by a

TABLE 3.—*Comparison of Constants*

Tissue	E_w' Extrapolated Potassium Substitution Potential, Mv.	u_K^0 Extrapolated Potassium Mobility	k', Mv./Eq.	c
Connective tissues (monkey, rat, capon)*	−3.7	1.37	+60	1.60
Pubic symphysis (guinea pig)†	−3.2	1.31	+51	1.80

* Data of Table 2.
† Joseph, Engel, and Catchpole.[6]

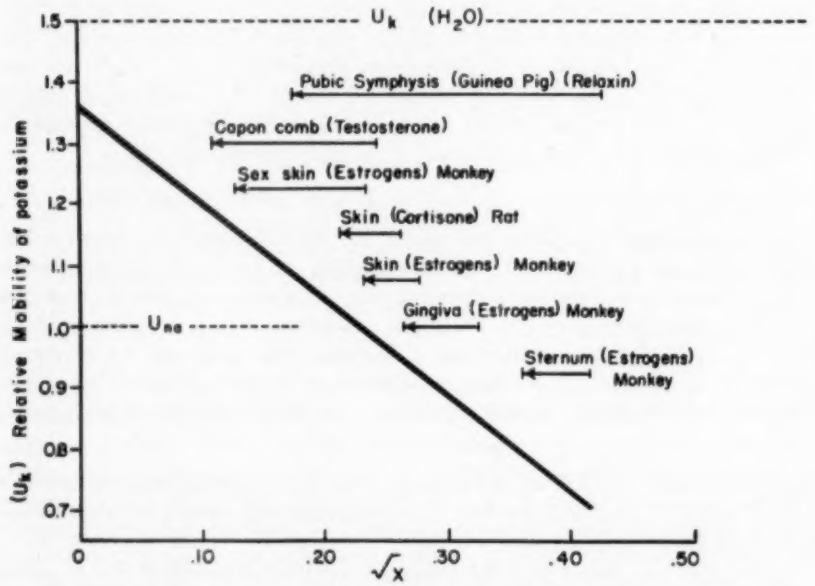


Fig. 6.—Relation of colloidal charge density, x, to potassium mobility, u_K, for several connective tissues. A square root relation is observed: ordinate, u_K; abscissa, square root of x. Upper and lower limits for each connective tissue are indicated. The effect of the hormone in each case is to decrease colloidal charge density and to increase potassium mobility.

mathematical method, which has been described.[6] These values are in good agreement with those found for the pubic symphysis of the guinea pig (Table 3). The experimental data are shown in Figure 1; the points have been fitted with two curves—one based on Equation 2, the other on Equation 3. Discrepancies between the two curves are less than 1 mv. The effects may thus be described in terms of the quantities u_K^0, c, E_w', and k', which have been found to have approximately the

same values for all the tissues studied (Table 3). The relation between potassium mobility and colloidal charge density in several tissues under hormonal influence is shown graphically (Fig. 6). In each case the effect of the hormone is to decrease x, the colloidal charge density, and to increase u_K, the relative potassium mobility. For each tissue upper and lower limits of x are indicated, the upper limit referring to the normal state, the lower showing the effect of hormone administration.

According to the data, the relative potassium mobility, u_K, decreases from about 1.4 to about 0.7 as x increases from 0 to 0.17 Eq. per kilogram of water. Assuming the mobility of sodium to remain constant, the mobility of potassium decreases by about one-half as the colloidal charge density varies from its lower to its upper limit. According to the theories of Debye and Hückel [32] and of Onsager,[31] there is a high correlation between mobility and activity coefficient in solutions where both are functions of the ionic atmosphere. Since Equation 3 is similar in form to Onsager's equation, such a correspondence will be assumed for connective tissues.

The equilibrium of sodium, potassium, and chloride between blood and connective tissues is indicated in Table 4, based on analytical results. In very loose con-

TABLE 4.—*Equilibrium Between Blood, Cartilage, and Synovial Fluid*

Tissue	Na	K	Ca (Ionic)	Cl	Na × Cl	K × Cl	Ca × Cl²
Synovial fluid*	139	3.8	1.7	102	14,180	388	17,600
Cartilage†	280	70	8.7	71	19,880	4,970	43,860
Blood‡	150	5	2.5	100	15,000	500	25,000

* Estimated from data of Ropes and Bauer [36]; concentrations in milliequivalents per liter.
† Data of Eichelberger, Brower, and Roma [37]; concentrations in milliequivalents per kilogram of water. See also [38].
‡ "Handbook of Biological Data." [39]

nective tissues, such as synovial fluid, the ionic products of sodium by chloride and potassium by chloride vary only slightly from the same ionic products in blood serum. Hence the activity coefficients of both salts may be assumed to be fairly constant in the two phases. In cartilage the product of sodium by chloride increases by a factor of about 1.3,§ while the product of potassium by chloride increases by a factor of almost 10 with reference to blood serum. If the effects are attributed to the activity coefficients, then the mean ionic activity coefficient of sodium chloride in cartilage is lowered only by about 15%. Since the product of potassium and chloride concentrations increases by a factor of almost 10 in cartilage, the mean ionic activity coefficient of potassium chloride may be assumed to decrease to about three-tenths its value in blood. The mean ionic activity coefficient is defined as the geometrical mean of the individual ionic activity coefficients. The chloride ion is common to both salts. Therefore, the effect indicates a highly selective lowering of the activity coefficient of potassium ion to a value of the order of one-tenth its value in blood serum. The effect on sodium is of a much smaller order of magnitude, as shown by the fairly constant value of the sodium-chloride ion concentration product. The results of the analytical distribution studies are in agreement with the interpretation given to the boundary potentials obtained with NaCl and KCl, that the mobility of potassium ion relative to that of sodium decreases in dense connective tissues to a value about one-half that found in aqueous solutions or in loose connective tissues [6] (Fig. 6). This interpretation is supported by the general nature of

§ References 13 and 14.

the relation between electrolyte activity coefficients and mobilities [31] in systems in which the properties of the ions are determined by coulombic forces. The distribution of sodium and potassium in ground substance is, according to this view, governed by the properties of the immobile colloidal anions, the aggregate of which functions as a cation exchange resin.‖ It may be recalled that Loeb long ago [33] postulated ion-colloid combinations, the changing proportions of which would alter the physical properties and irritability of tissues. A similar assumption has been made in the fixed charge hypothesis of Ling [34] as an interpretation of sodium-potassium exchange in muscle and nerve. The exchange resin principle may thus be applicable to tissues and biological structures of diverse nature.

In order to describe the thermodynamic properties of ground substance as an exchange resin for sodium and potassium, the equilibrium data of Table 4 may be applied. From the data, an estimation of the free energy of sodium-potassium exchange is possible.

The equilibrium distribution of the ions between blood serum and articular cartilage is given by

$$K_s' + Na_{ms}'' = Na_{ts}' + K_{ts}'' \qquad (4)$$

where

$$\Delta F = 0$$

The reaction represents the exchange of an equivalent of blood potassium ions, K′, for an equivalent of cartilage sodium, Na″.

The numerical subscripts denote the equilibrium values of the several ionic concentrations in milliequivalents per kilogram water. Since the concentrations refer to the equilibrium distribution given in Table 4, in which the chemical potentials of NaCl and KCl are assumed constant, the free energy change, ΔF, is assumed to be zero.

The standard free energy change, ΔF^0, of the potassium-sodium exchange reaction may be calculated in the usual way from the free energies of transferring the ions to a standard state of a given concentration in either phase. Then

$$\Delta F^0 = -RT \ln \frac{(Na') \ (K'')}{(Na'') \ (K')}$$

$$\Delta F^0 = -RT \ln \frac{(150) \ (70)}{(280) \ (5)}$$

$$= -1{,}250 \text{ Cal. } 37 \text{ C.}$$

where R is the gas constant, T the absolute temperature, and ΔF^0 is the standard free energy of the exchange reaction

$$K' + Na'' = Na' + K'' \qquad (5)$$

under conditions where each ion is at the same concentration (0.15 Eq. per kilogram of water). Under standard conditions, therefore, there is a strong tendency for potassium to displace sodium from the colloidal phase, and this is reflected in the highly asymmetrical distribution of the two ions between the two phases (Table 4; Reaction 4).

The standard free energy of potassium-sodium exchange may be compared with that of calcium-sodium exchange. This reaction may be represented to include the immobile anionic aggregate X,

$$Ca^{++} + Na_2X = CaX + 2Na^+ \qquad (6)$$

All the ions may be considered to occur within the colloid-rich phase, in equilibrium

‖ References 6 and 27.

with blood and the water-rich phase. The colloidal calcium complex has been found to be largely undissociated, while the sodium salt of the colloid is assumed to be completely dissociated. Accordingly, ΔF^0 may be obtained from the equilibrium,

$$CaX = Ca^{++} + X''$$

where

$$K = 0.026.¶$$

The standard molal free energy of this dissociation is

$$\Delta F^0 = -RT \ln (0.026)$$

For Reaction 6, therefore, the standard free energy change is $\Delta F^0 = -2{,}300$ Cal., and calcium, like potassium, has a strong tendency under standard conditions to displace sodium from the colloidal anionic groups. With calcium, however, undissociated complexes appear to be formed, so that only a fraction of the calcium is ionic.#

The mobility of the free calcium ions is apparently not influenced by the density of colloidal charges,[6] Potassium, on the other hand, appears to be selectively attracted by strong electrostatic forces, an effect which is manifested electrometrically by lowered relative mobility of the ions, and analytically by high concentrations in connective tissue.

From the standard free energies of Reactions 4 and 6, the free energy of potassium-calcium exchange may be obtained. Thus, for the reaction

$$Ca^{++} + K_2X \rightleftharpoons CaX + 2K^+ \qquad (7)$$

$$\Delta F^0 = 200 \text{ Cal.}$$

This figure differs by about 2,500 Cal. from the constant for the sodium-calcium reaction. Potassium ion, as compared with sodium ion, thus tends to inhibit the formation of the colloidal calcium complex. Sobel [35] has found that potassium ion has a strongly inhibiting effect on in vitro calcification of epiphyseal slices, with sodium antagonizing the potassium effect. His results may be interpreted as a tendency of potassium to inhibit combination of calcium with colloid, as shown by the positive standard free energy of Reaction 7. From the value of the free energy, the equilibrium constant K can be computed in the usual way, from the known value of $RT \ln K$. Thus, when the colloid is neutralized by potassium rather than by sodium, $K = 1.4$. In the presence of sodium, the value of K is about 0.026. The value thus varies by a factor of about 50 when potassium is substituted for sodium.

The free energies of Reactions 5, 6, and 7 yield equilibrium distributions of the ions between blood and connective tissue colloids. For constant blood sodium and potassium, the distribution of electrolytes in the system has been represented by a nomogram in which the independent variables are blood ionic calcium and the density of colloidal charge.* In physiological systems, the variables must include sodium, potassium, calcium, and magnesium, all of which react in some manner with the colloidal components. The variations in blood may be represented by taking the ions in pairs, varying any two, and holding the others constant. Omitting magnesium ion from the calculations, it is possible to represent the system as follows: colloid, sodium, potassium, and calcium, taking the independent variables

¶ References 13 and 14.

References 13 and 14.

* References 13 and 14.

two at a time. The distribution of calcium and sodium between blood and connective tissues has been represented in this manner by a nomogram in which blood calcium and the density of colloidal charge were taken as the independent variables.

The effects of independent variations of the ions at a constant value of colloidal charge density (that of normal monkey sternum) are shown in Figure 7. Three nomograms are included, in each of which one of the ions is taken at its normal blood level and the other two varied above and below their normal blood levels. Five scales are represented in each nomogram: the concentrations of the two independently variable blood ions and the concentrations of the three ions in connective tissue of the given state of colloidal aggregation. The connective tissue concentrations, $(Na)'$, $(K)'$, and $(Ca)'$, are conveniently regarded as dependent variables, while the blood concentrations, $(Na)^o$, $(K)^o$, and $(Ca)^o$, represent the independent

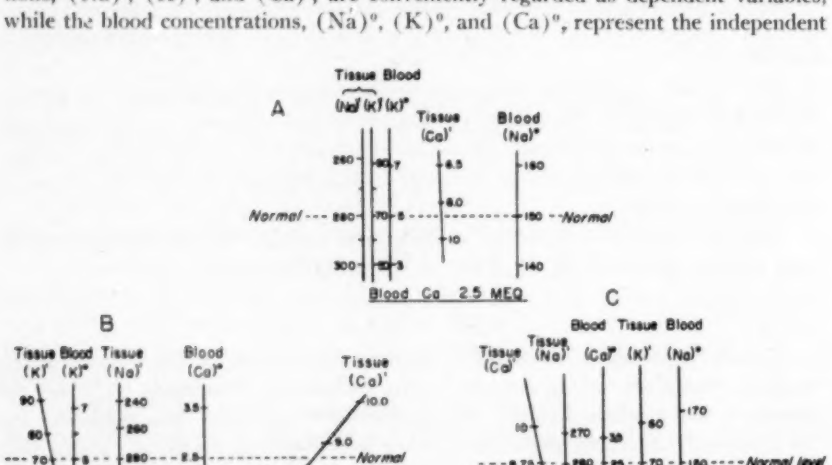


Fig. 7.—Ionic exchange nomograms for cartilage. The properties are derived from the standard free energies of exchange of potassium and calcium for sodium (Equations 5 and 6). Horizontal lines represent normal homeostatic distribution of ions between blood and cartilage (Table 4). Six variables are represented as follows: tissue sodium, potassium, and calcium, $(Na)'$, $(K)'$, and $(Ca)'$ in milliequivalents per kilogram of water; and serum sodium, potassium, and calcium, (Na), (K), and (Ca) in the same units. The calcium denotes the ionized fraction. In each nomogram, one of the blood values is held constant at the normal level. Thus, all states are represented in which any two of the blood ionic levels are nonhomeostatic.

variables. The nomograms thus represent all states in which two of the blood ion levels are independently variable and in which the third ion level is at its normal physiological value. The horizontal dotted lines represent the normal (homeostatic) distribution of the ions, in which all three ions are at the assumed normal levels.

Increase of the blood level of any one of the ions increases the tissue concentration of that ion and decreases the tissue concentrations of the other two. A decrease of the blood level produces the reverse effects. Such an alteration of blood level for one ion can be read on either of two nomograms. Thus variations of blood potassium at normal levels of sodium and calcium may be read equally well on either nomogram *A* or *B* (Fig. 7). It follows that the alterations of tissue composition

produced by increasing the level of any one ion may be at least partially reversed by increasing the level of either one of the other two ions. Thus, if ion *A* displaces ions *B* and *C* from the tissue, a partial compensation is obtained by an increase of the blood level of either *B* or *C*. Then, an increase of *B* may partially restore the initial concentrations of both *A* and *B*, but at the cost of further decreasing the concentration of *C*. Similarly, a restoration of the initial level of *C* implies a decrease of *B*. Reactions 4, 6, and 7 operate simultaneously, and each is characterized by a different standard free energy change and equilibrium constant. It has been shown above that these constants are quite different for the three reactions; it therefore follows that the displacement effects are asymmetrical, and this is clear from the nomograms. From Nomogram A, it is evident that a small increase of blood potassium produces a large increase of tissue potassium, a large decrease of tissue sodium, and a decrease of tissue calcium. An increase of blood potassium, for example, from 5 to 5.5 mEq. produces changes of tissue sodium and potassium that cannot be reversed by any increase of sodium on the scale of the diagram. However, an increase of blood sodium to 165 mEq. (10% above normal) produces only small changes in ground substance potassium and calcium, and these small decreases can be reversed by small increases of the blood levels. Thus, potassium and calcium are characterized by their high affinities for the colloid, and sodium becomes the most readily displaceable ion of the connective tissue. The resulting ionic antagonisms are highly asymmetric. Thus from Nomogram *B*, it is evident that small increases of blood potassium strongly affect tissue potassium, sodium, and calcium, but that blood calcium level has much less effect on tissue potassium than on tissue sodium.

The inorganic composition of the colloidal matrix of connective tissue is thus responsive with varying sensitivity to changes of the blood electrolytes, and is determined by the concentrations of all diffusible ions which can enter the matrix and react in some manner with the colloid. Any change of the blood electrolytes results in a change of equilibrium which can be only partially compensated by any other modification that does not restore the blood electrolytes to their initial state.

HOMEOSTASIS IN HETEROGENEOUS SYSTEMS

The total potassium content of connective tissues, like the total calcium content, depends on the state of aggregation of the colloids of the matrix. Loose connective tissues, in which the water content is high, and which contain small amounts of insoluble, highly aggregated colloid, have potassium and calcium concentrations that approach those of blood. In cartilage, on the other hand, potassium concentration is more than 10 times that of blood, while total calcium is concentrated almost to the same extent. Both effects depend on high free energies of formation of the potassium and calcium colloidal complexes from the corresponding sodium complex. They are thus consonant with the Gibbs-Donnan equilibrium, which depends on invariant chemical potentials of all the diffusible electrolyes and of water. Physiological homeostasis within the heterogeneous colloidal matrix is thus more clearly identifiable with constant chemical potentials than with total electrolyte concentrations. These chemical potentials are the same as those of the diffusible electrolytes and water of blood, so that homeostasis depends essentially on invariance of the blood electrolyte concentrations.

It has previously been explained how the macroconcentrations of sodium, calcium, colloid, and water may vary over wide limits independently of the microcon-

centrations of the various submicroscopic coexistent phases of the colloidal matrix.† The same reasoning applies to potassium. If the microconcentration, C_K'', of potassium within the colloid-rich phase is 70 mEq. per kilogram of water, and the microconcentration, C_K', within the water-rich phase is 5 mEq. per kilogram of water, the macroconcentration, C_K, may vary between these two limits under conditions in which all the microconcentrations are constant. This is possible only if the composition of blood also remains constant. Thus the extensive properties of the matrix may vary independently of the intensive properties. The changes induced by hormones seem typically to lower the density of colloidal charge, resulting in decrease of base binding of the tissue, increase of the water content, and increase of the ratio of soluble to insoluble colloid. Concomitantly, there occur striking alterations in such mechanical properties as viscosity and plasticity. But, insofar as variations in blood electrolyte composition are not induced, the changes are consonant with physiological homeostasis. They depend on variations of the extensive properties of the system, such as the density of colloidal charge, which is conveniently taken as the independent variable. This determines the macroconcentrations of all the components, which may vary independently of the microconcentrations and the chemical potentials. Functional adaptations, such as those induced by hormones, depend on changes in the state of aggregation, which determines the distribution of the colloid between water-rich and colloid-rich phases.

SUMMARY

The interaction of sodium, potassium, and other ions with connective tissue has been studied by an electrometric method, that of determining substitution potentials at a liquid junction in the tissue. The potentials observed have been found to depend on the charge density of the colloidal matrix of the connective tissue. Besides using normal tissues, these were also subjected to the action of various hormones whose general effect is to cause a disaggregation of the colloidal matrix, accompanied by changes in electrolyte distribution and water uptake from the blood. The following tissues and hormone combinations were used: symphysis pubis of the guinea pig (estrogen, relaxin); capon comb (testosterone propionate); macaque monkey sex skin, skin, and gingiva (estradiol benzoate), and rat skin (cortisone). Histochemical studies were made on all normal and hormone-treated tissues, fixed by freezing-drying, and stained for carbohydrate-containing proteins. The findings were consistent with the explanation of glycoprotein disaggregation and formation of water-soluble fractions as important features of hormone action on connective tissue. Potassium is selectively immobilized by dense connective tissues, which consequently act as ion exchange resins for potassium. In normally loose connective tissues, or in those loosened by hormone action, the colloidal charge density is decreased. In consequence, the relative mobility of potassium ion as compared with sodium increases from its value of 0.7, in dense tissues, to 1.4, in loose tissues, the latter approaching its value in water.

The Gibbs-Donnan equilibrium is used to describe the reaction of ions with an indiffusible, negatively charged matrix. A consideration of the thermodynamic properties of ground substance shows a strong tendency for potassium to displace sodium from the colloidal phase; the standard free energy of the exchange is

† References 13 and 14.

— 1,250 Cal. at 37 C. Calcium also tends to displace sodium, with a ΔF^0 of — 2,300 Cal. Derivation of the standard free energy of the potassium-calcium exchange shows a ΔF^0 of 200 Cal., indicating that potassium ion tends to inhibit the formation of the colloidal calcium complex. These reactions have been generalized in a series of nomograms which show the strongly asymmetrical character of the interactions of sodium, potassium, and calcium with the ground substance and with each other. The nomograms describe the conditions for physiological homeostasis of the several tissues with blood.

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ATYPICAL PROLIFERATION OF BRONCHIOLAR EPITHELIUM

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IN RECENT years considerable interest has developed over peculiar small cellular proliferations in the lung. These cell aggregates were noted as incidental findings in a very wide variety of autopsy material and, in addition, in lobectomies or pneumonectomies in which operation was performed for bronchiectasis or cystic disease of the lung. There has been reasonable agreement over the essential similarity of the various reported cases but considerable difference of opinion regarding the interpretation of the lesions.

Womack and Graham [1] studied nine cases of congenital cystic disease of the lung. In three instances they found an abnormal epithelial overgrowth, consisting of "poorly differentiated epithelial cells . . . showing a definite tendency toward invasion but nowhere presenting any evidence of metastasis." These authors entitled their paper "Metaplasia in Cystic Disease of the Lung," and did not consider the process malignant "from a clinical standpoint." On the other hand, Stewart and Allison,[2] with a case seemingly identical, believed they were dealing with a carcinoma of peculiar type, resembling basal-cell carcinoma of the skin. They suggested a possible basal-cell carcinomatous development from squamous metaplastic epithelium.

These lesions occur in bronchiectasis, but also in other conditions. Small tumor-like growths in the lungs have been described and regarded as early carcinoma by Gray and Cordonnier [3]; Peterson, Hunt, and Sneedon [4]; Horrell and Howe,[5] and Spain and Parsonnet.[6] Probably one case described by Karsner and Saphir [7] is also in this category. Spain and Parsonnet describe lymph node metastasis in their case, but no other author has made this claim.

Prior and Jones,[8] and later Prior,[9] added 20 new cases and analyzed and summarized the preceding examples. They believe the lesions are neoplasms, but benign. They point out that the age and sex distribution is quite different from that in bronchogenic carcinoma. They believe there is similarity to bronchial adenomas of the carcinoid type. The origin of the proliferation, they believe, is from the "terminal bronchiole." Gray and Cordonnier had already maintained an origin from the "alveolar ducts." Prior and Jones, and Prior drew a distinction between proliferation of a squamous character and the spindle-shaped cell multiplications. Such a distinction, in my opinion, is not valid, for reasons that are discussed subsequently.

The situation is somewhat complicated by reports * of carcinoma in situ in major bronchi, proved by bronchoscopic biopsies. It is probable that these cases are not in the same category as the bronchiolar proliferations discussed herein.

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* References 10 and 11.

In our series of 1,450 consecutive autopsies in a general hospital, 15 instances were found which reveal proliferation of epithelium of peculiar character in the distal portions of the bronchial tree. In some cases the cell masses can be traced to the epithelium of small bronchi, bronchioles, and atria. In other cases, with cells filling up alveoli, a direct connection to bronchiolar or atrial epithelium cannot be demonstrated, but is presumptive. In some cases the cells show a squamous configuration. In all instances the lung findings were chance microscopic observations, and in no case was change suspected on gross examination. The cases are very briefly summarized.

Case 1.—This 77-year-old woman died of subacute granulomatous pericarditis, which resembled tuberculosis, although tubercle bacilli could not be demonstrated, nor could any active focus of tuberculosis be found elsewhere in the body. The lungs, weighing 460 and 300 gm., showed only acute and chronic passive congestion, with recent infarcts, and focal atelectasis. There was no evidence of active tuberculosis. Subsidiary findings of interest included a well-encapsulated hypernephroid adenoma of the kidney, which did not invade the surrounding parenchyma, and a benign papillary cystoma of one ovary.

Numerous foci of atypical proliferation were found in the lung in severely atelectatic tissue close to a recent infarct. In the viable tissue at the margin of the infarcted zone there is proliferation of cells, spindle-shaped and occasionally squamous, which line or fill the compressed alveolar spaces. The cells seem to arise from terminal bronchioles and to spread by contiguity from one alveolus to another.

Case 2.—This 87-year-old woman died after a three-month stay in the hospital following an old fracture of the neck of the right femur. The major pathologic diagnoses were generalized arteriosclerosis, myocardial fibrosis, senile amyloidosis of the heart and lungs, Alzheimer's change in the brain, with senile plaques, and recent fresh infarct of the lung. The lungs weighed 420 and 360 gm. At the extreme margin of an infarct there is a small zone of atypical proliferation of epithelium. Several compressed aveoli are filled with masses of small cells, closely compacted, with regular, even nuclei, somewhat elongated and spindle-shaped. There are several small clusters which are present in seemingly disconnected alveoli. However, the absence of serial sections prevents a definite statement whether connection by contiguity is present.

Case 3.—This 78-year-old white man showed the following major pathologic conditions. The heart had undergone massive hypertrophy, weighing 890 gm. Arteriosclerosis of the coronary arteries was relatively severe. There was neither hypertension nor valvular disease, and the diagnosis of idiopathic hypertrophy was made. In addition, there was a moderate degree of hydrothorax, with atelectasis. Subsidiary findings include a nodular hyperplasia of the prostate and chronic cholecystitis with cholelithiasis. The lungs weighed 450 gm. each. The atypical proliferation of bronchial epithelium in this case is found in zones not related to atelectasis. In the angle formed between a blood vessel and a bronchus a cluster of alveoli is virtually filled with proliferated cells of regular appearance, somewhat oat-shaped in configuration with poorly defined cellular boundaries. The nuclei are entirely regular and benign in appearance. Only one single focus was found, in the extreme corner of one block. Other sections, which reveal definite atelectasis, do not show this type of proliferation.

Case 4.—This 80-year-old man, with severe generalized arteriosclerosis, died of multiple abscesses of the lung, of aspiration type, and very severe confluent bronchopneumonia. Subsidiary findings of interest included healed pulmonary tuberculosis, benign nodular hyperplasia of the prostate, and a small carcinoma of the prostate with proliferation of atypical cellular forms that invaded the capsule, but altogether to a very small extent. The lungs weighed 975 and 915 gm. The gangrenous cavities varied from 1.5 to 7 cm. in diameter. Besides the extensive gangrene there was marked atelectasis and severe bronchopneumonia and bronchitis, together with chronic passive congestion and fibrotic changes. There are numerous scattered areas of proliferation of cells, which show a generally squamous configuration and are situated in close relationship to the adventitia of blood vessels. The cells only partly fill the alveoli. They seem to stream from one alveolus to another. Out of a dozen blocks of lung tissue, only three show this change.

CASE 5.—This 70-year-old woman died of a carcinoma of the parotid gland, with very extensive local spread in spite of previous radical neck dissection. There were no distant metastases. The immediate cause of death was bronchopneumonia. The lungs, weighing 450 and 390 gm., showed severe atelectasis, with fibrous thickening of the septa and abundant acute bronchitis, with chronic inflammatory changes in the septa. In one single area small air spaces are virtually filled with proliferating epithelium, in part squamous, in part of uniform but

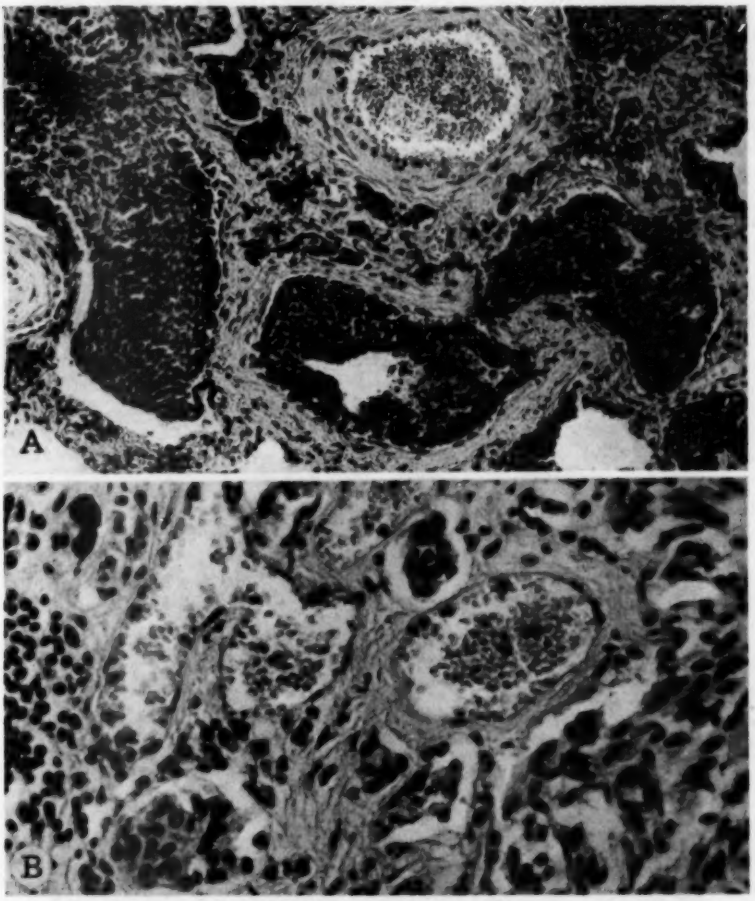

Fig. 1.—*A* (Case 9), multiplication of spindle-shaped cells in alveoli adjacent to the fibrous adventitia of an artery. Small cell clusters are present in spaces that suggest lymphatics.
B (Case 13), cells in possible lymphatics.

undifferentiated character. Similar masses of mixed oat-cell and squamous-cell appearance, are also present in a few adjacent alveoli, which are only partially filled.

CASE 6.—This 71-year-old woman entered the hospital in terminal condition with an adenocarcinoma of the sigmoid colon metastasizing to regional lymph nodes, severe diffuse hemorrhagic colitis, marked emaciation, and fatty metamorphosis and central necrosis of the liver. The lungs weighed 400 and 250 gm. The lower lobe of the left lung showed marked fibrosis, with saccular dilatation of bronchi; many cystic spaces filled with a protein coagulum; many large,

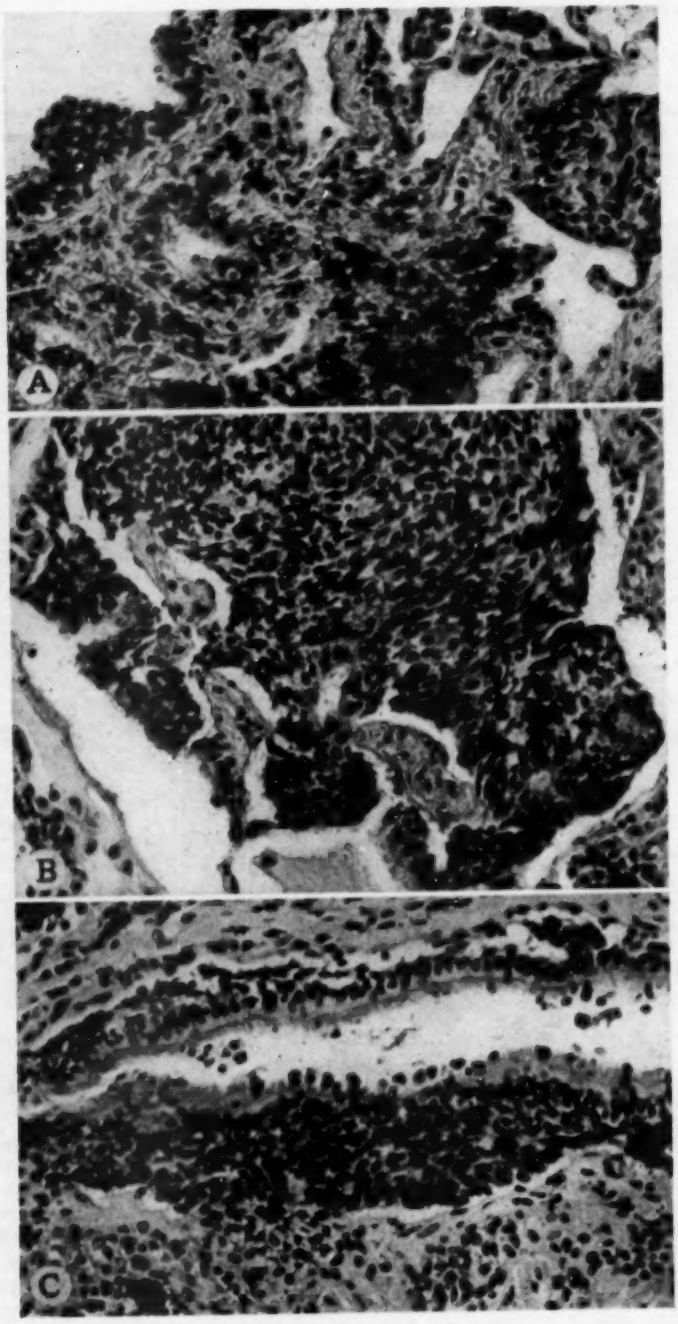

Figure 2

(See legends on opposite page)

very thick-walled blood vessels, and severe atelectasis of persisting alveolar tissue. Our interpretation of this lesion is that of a localized cystic disease of the lung. Microscopic examination (Fig. 5) reveals innumerable foci of atypical cellular proliferation in compressed alveoli and adjacent fibrous tissue. The cell masses, which are regular in appearance, fill alveoli in whole or in part and can readily be seen streaming from one alveolus to another and spreading peripherally. In some areas they arise from a clearly defined bronchial or bronchiolar epithelium and spread into adjacent alveoli.

CASE 7.—This 50-year-old woman suffered from severe rheumatic heart disease. The pathologic diagnoses were healed rheumatic valvulitis, mitral and aortic; chronic passive congestion of lungs; severe recent and old infarcts of lungs; chronic passive congestion of liver, with early cardiac cirrhosis; mural thrombus, left auricle; recent encephalomalacia, and congestion o flungs; severe recent and old infarcts of lung; chronic passive congestion of liver, In addition to the severe chronic passive congestion, there were several small infarcts, about 3 cm. across. At the margin of one of these, directly adjacent to the wall of a thrombosed artery, there is a single focus of proliferation, with small masses of squamous type epithelium that fill partly compressed alveoli. In the center of these cell masses there are granules of hemosiderin, similar to the hemosiderin which is found widely dispersed in adjacent lung tissue.

CASE 8.—This 86-year-old woman was admitted with clinical signs of a cerebrovascular accident. However, since the permission for autopsy expressly forbade the examination of the head, only the visceral organs were examined. The pathologic diagnoses included brown atrophy of the heart; thrombus in the right pulmonary artery, but without infarction; congenital cysts of the liver, and an acute peptic ulcer of the stomach. There was only moderate arteriosclerotic change. The lungs weighed 240 and 175 gm. and showed emphysema, patchy atelectasis, and small zones of fibrosis. Two areas of atypical proliferation were noted. One centered around a small scar where there was multiplication of spindle-shaped cells within the connective tissue, involving adjacent bronchioles and alveoli (Fig. 2*A* and *B*). Some of the peculiar histologic details are discussed at greater length below. A second, independent lesion arose in relation to a small bronchiole near the adventitia of a larger bronchus (Fig. 2*C*).

CASE 9.—This 62-year-old woman suffered from diabetes mellitus and severe hypertension. The pathologic diagnoses were clinical diabetes mellitus; generalized arteriosclerosis and arteriolosclerosis; arteriolosclerotic nephrosclerosis, severe (clinical hypertension); myocardial hypertrophy; focal myocardial fibrosis; chronic passive congestion of lungs; hydrothorax, bilateral; pulmonary atelectasis, and fibrinous pericarditis (clinical uremia). Subsidiary diagnoses included a well-marked nodular hyperplasia of the thyroid, with fibrosis and exhaustion changes, and a cortical adenoma of the adrenals. The lungs weighed 310 and 250 gm. In one area, not related to any atelectasis, there is a zone of cellular proliferation around a medium-sized artery (Fig. 1*A*). This proliferation fills numerous alveoli and spreads from one alveolus to another. As the cells enter a new alveolus, they tend to spread around the margin and to form a lining. They then proliferate and fill up the lumen. In the process of filling the alveoli the cells surround hemosiderin granules that are already present. Small masses of proliferating cells lie within the connective tissue adventitia of the blood vessels and seem to be in tissue spaces or possible lymphatics.

CASE 10.—This 73-year-old man suffered from arteriosclerotic heart disease. The major pathologic diagnoses were arteriosclerosis of coronary arteries; myocardial hypertrophy; old thrombosis of right coronary artery, with healed myocardial infarct; chronic passive congestion of lungs; small infarct of lung; hydrothorax, bilateral, and atelectasis. The lungs weighed 420

EXPLANATION OF FIGURE 2

Fig. 2.—(Case 8) *A*, on the left, proliferation of basal type cells, which underlie the ciliated epithelium of a small bronchiole. One the right is a solid mass of cells partly filling an alveolus.

B, mass of proliferating cells in intimate relation with a ciliated epithelial layer. Note the spread of cells across or through alveolar walls.

C, extensive proliferation of cells from the basal elements of a bronchiole. Note the various foci of multiplication. This bronchiole is completely surrounded by abundant connective tissue and does not abut on alveoli, as occurs in *A* and *B*.

and 390 gm. There was a single zone of fresh infarction, 2.5 by 1.5 by 1 cm., in the left lower lobe. At the margin of the infarct, where there is considerable atelectasis, are masses of proliferating epithelium which fill compressed alveoli, extending from one alveolus to another. The cells appear to arise in part from bronchiolar epithelium and frequently exhibit a squamous

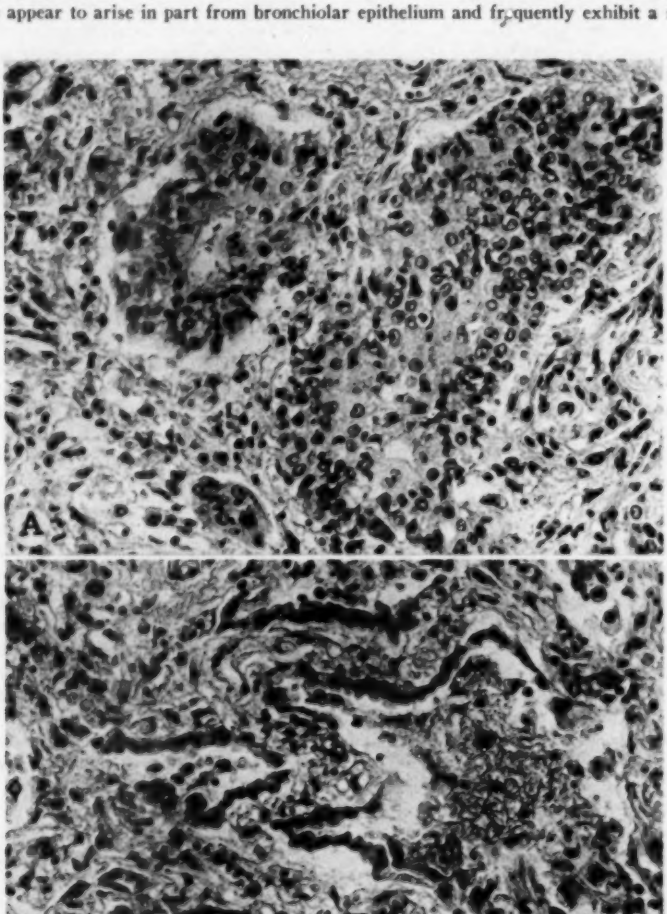

Fig. 3 (Case 10).—*A*, multiplication of cells, predominantly squamous in type, within compressed alveolar spaces. Note, in the upper left corner, the simple cuboidal lining. Compare with *B*.

B, a microscopic field immediately adjacent to that shown in *A*. This paper maintains that the changes in *A* develop from these in *B*.

character (Fig. 3*A*). In this area of lung, where the atelectasis is severe, there is a cell proliferation of much more banal and entirely familiar type, namely, the covering over of alveolar walls by a single layer of plump cuboidal cells (Fig. 3*B*).

CASE 11.—This 40-year-old man had severe arteriosclerotic heart disease. The pathologic diagnoses were arteriosclerosis of coronary arteries, severe, with old occlusions; myocardial hypertrophy and fibrosis; recent occlusion of a coronary artery, and acute pulmonary edema. Changes of acute myocardial infarction were not present. The lungs weighed 800 and 900 gm. There was marked pulmonary edema but no other significant gross changes. Microscopically, a single focus is found where, surrounding a small thin-walled vein, there are masses of proliferating epithelium of spindle-shaped type. These cells seem to arise from a cuboidal or low columnar type of epithelium which lines respiratory bronchioles or atrium, and then to fill up alveolar spaces and extend into neighboring alveoli. The total amount of cellular proliferation is very small, and the focus is minute. There is no relationship to any area of fibrosis or scarring, atelectasis, or infarction.

CASE 12.—This 77-year-old man suffered from marked arteriosclerosis, with rupture of the abdominal aorta. Additional diagnoses included hyalinization of the islands of Langerhans (clinical diabetes mellitus); nodular hypertrophy of the prostate, with hydroureter and hydronephrosis, bilateral; primary senile amyloidosis of the heart; slight chronic pancreatitis with fat necrosis; early carcinoma of stomach, and focal necrosis of liver. The carcinoma of the stomach was a small polypoid mass with a narrow stalk, measuring altogether 3 by 2 by 2.5 cm. The amyloid in the heart was of relatively slight degree, and the heart weighed only 320 gm. The right and left lungs weighed 350 and 260 gm. respectively. There were areas of emphysema and atelectasis, distributed in patchy fashion. Microscopically, adjacent to an area of fibrosis, there are masses of small oat-cell proliferations that line or fill up many alveoli at the margin of the fibrotic zone. The cells appear to arise directly from the epithelium of the terminal bronchioles. Some cell masses are present within the fibrotic zone.

CASE 13.—This 74-year-old woman suffered from arteriosclerotic heart disease as a major complaint. The pathologic diagnoses were generalized arteriosclerosis; arteriosclerosis of coronary arteries; hypertrophy of heart, with diffuse fibrosis; chronic passive congestion of lungs; healed myocardial infarct, and bronchopneumonia. As a separate disease sequence, she had chronic myelogenous leukemia, involving the spleen, liver, and bone marrow. A further disease sequence consisted of severe scarring and contraction of the kidneys (but without clinical hypertension), the origin of which was not clear, but which may have been an old pyelonephritis. Terminally, a diffuse fine nephrolithiasis was present in both kidneys. The lungs weighed 430 and 250 gm. and showed patchy pneumonia and moderate chronic passive congestion. There are two special areas, 3 to 4 mm. across, grossly obscured by the bronchopneumonic foci, which microscopically show masses of spindle-shaped cells, in part surrounded by fibrous stroma, wherein pulmonary architecture is no longer recognizable, but in part invading the adjacent marginal alveoli (Fig. 2). In the fibrous parts of the growth the proliferating cells seem to lie within lymphatics. In nodules of this size the origin of these cells cannot be satisfactorily established.

CASE 14.—This 86-year-old woman died with generalized arteriosclerosis, pulmonary congestion, edema, and bronchopneumonia. The lungs, weighing 450 and 300 gm., showed, in addition, chronic passive congestion, recent infarction, and atelectasis with fibrosis and organization. In the areas of atelectasis and fibrosis the compressed spaces are frequently lined by epithelial cells of varying type, flattened, cuboidal, columnar, spindle-shaped, and squamous. In some alveoli (or terminal bronchioles) the lining is one cell in thickness, but in others the lining is multilayered. Some spaces are completely filled with proliferated cells (Fig. 4*A*).

CASE 15.—This 80-year-old man, with previous transurethral resection for carcinoma of the prostate, died with extensive local recurrence and metastases to the bone, liver, and lungs. Other diagnoses included generalized arteriosclerosis with myocardial hypertrophy and fibrosis; bilateral hydrothorax; pulmonary atelectasis, and thrombi in pulmonary arterial branches, but without infarction. The lungs weighed 300 and 210 gm. In the atelectatic tissue (Fig. 4*B*) many of the compressed alveoli are lined by flattened or low cuboidal cells, sometimes one layer in thickness, but sometimes several layers deep. The proliferation seems to be the atypical continuation of cuboidal bronchiolar epithelium, extending downward into the compressed and relatively fixed air sacs. In some zones the proliferated cells assume a suggestively squamous appearance. The few foci of metastatic carcinoma of the prostate are distinguishable with the greatest ease from these atypical bronchiolar proliferations.

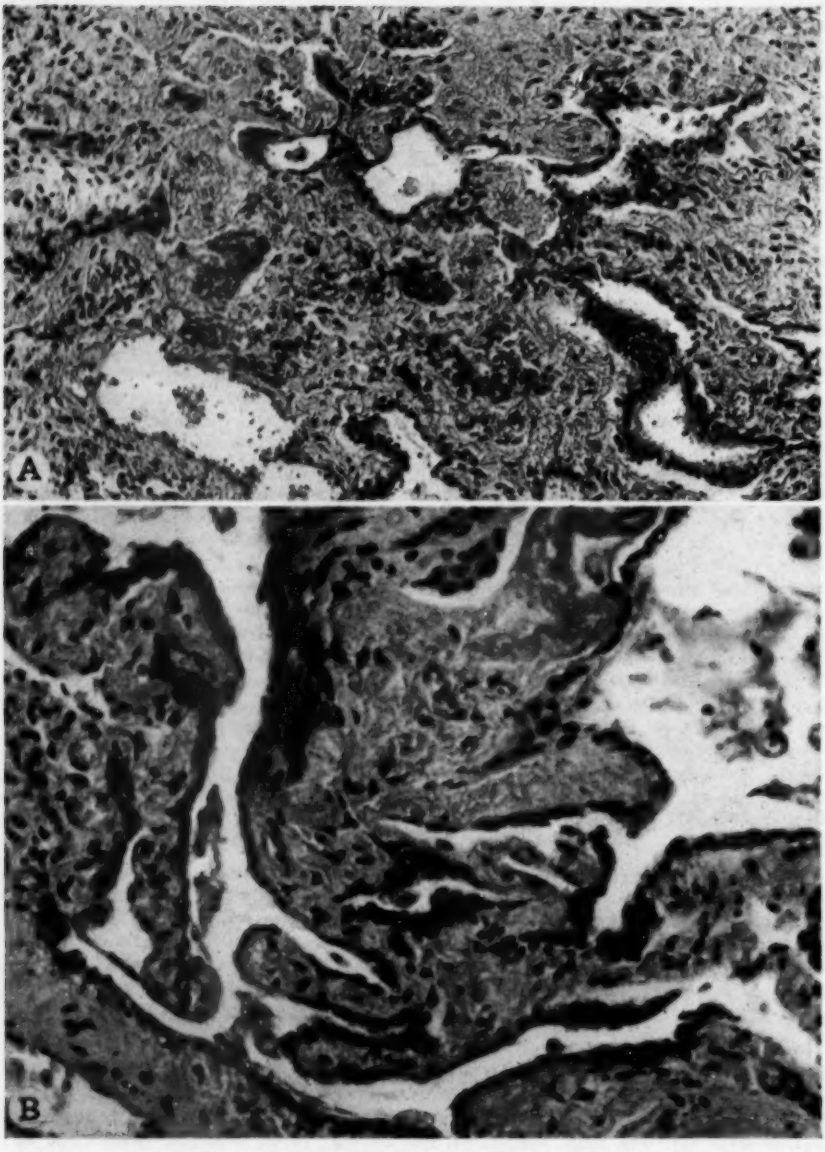

Fig. 4.—*A* (Case 14), squamous metaplasia and proliferation filling alveolar spaces. Note the atelectasis and fibrosis.

B, proliferation of spindle-shaped cells lining the surfaces of compressed alveoli and heaping up into small solid masses and clusters.

PATHOLOGIC CHANGES

A study of our pathologic material brings out certain distinct features. In Figure 1*A* (Case 9) one sees an over-all view of a well-developed characteristic lesion. The proliferations occur immediately around an area of relative fixation in the lung, which in this case is the fibrous tissue surrounding a small artery. In other cases the fixation may be from atelectasis, bronchiectasis, or the immediate presence of infarcts or abscesses. The cells are spindle-shaped or oat-cell in appearance, are very regular, and tend to fill the involved alveoli. In other cases, the main mass of proliferating cells may show varying amounts of squamous differentiation. Cells stream from one air sac to another. On entrance into a new alveolus they first spread out along the margins and then fill the lumen. Small cell masses exist in connective tissue spaces, which may represent lymphatics but are not unequivocally demonstrated as such. A field from Case 13 is seen in figure 1*B*, which shows, under higher power, the cells in putative lymphatics, as well as in large solid masses.

The origin of the cells occasions some difficulty. Previous observers have traced them to the basal layer of the bronchiolar epithelium. We also have seen evidence which would support such a claim. In figure 2*A*, for example (Case 8), is seen, in a small terminal or respiratory bronchiole, a heaped-up mound of cells beneath the columnar surface epithelium. This focus occurs immediately adjacent to a zone of fibrosis, and there can be little doubt that the proliferated cells represent a multiplication of basal elements. Similarly, in Figure 2*C*, from the same case, a larger bronchiole shows more extensive proliferation in several discrete foci, with extension into the underlying connective tissue.

It does not necessarily follow, of course, that a contiguity proves an origin. In Figure 2*B*, also from the same case, a streaming of cells is seen from the large mass in the center to the bronchioles overlying. It is possible that the cells are invading the surface epithelium from below, rather than the reverse. Nevertheless, the study of our material yields substantial assurance that one of the modes of origin is a growth from the relatively undifferentiated cells that form a basal layer in the small bronchioles.

We are convinced, however, that this is not the only mode of development for these atypical proliferations. To appreciate an alternative mode, we must stress the character of the material: Of the 15 cases in our series, 11 showed pulmonary infarcts, or a high degree of atelectasis, or pulmonary fibrosis, or a combination of any two or three of these. An additional two cases showed chronic passive congestion without infarction, atelectasis, or fibrosis. There were only two cases in the entire series in which the atypical proliferations occurred in a lung without any of these other features. This recalls the remark of Peterson, Hunter, and Sneedon, who stressed the occurrence in their cases of bronchiectasis, bronchitis, or focal pulmonary fibrosis.

It is our belief that in large part these peculiar changes, described above, are related to the more banal proliferation of epithelium seen in a lung where fibrosis, or other causes, limits the expansion of tissue. It is a common finding to see alveoli adjacent to a scar, for example, lined by a single layer of plump cuboidal cells. Some authorities consider these cells as alveolar epithelium. Other pathologists, with whom I concur, consider these cells a downgrowth, into alveoli, of epithelium from very terminal bronchioles. Somehow, for reasons not clear, a focal zone of

poor alveolar expansion promotes an epithelization of a surface not originally covered by epithelium. The illustrations of Stewart and Allison show, very strikingly, this type of cuboidal epithelium, in proximity to the spindle-cell proliferations.

In the present series of cases there are many suggestions that this fundamental pathogenesis is applicable, albeit with certain modifications. In Figure 3*B* (Case 10), for example, in an area of pronounced atelectasis, are seen single layers of cuboidal cells lining the alveolar surfaces. These cells are morphologically very different from the proliferations figured elsewhere. The plump cuboidal cells occasionally heap up into clusters. Figure 3*A*, which is an adjacent field from the same slide, shows a transition between a single layer lining a surface and solid masses which fill alveolar spaces. In Figure 6 the cells have a definitely squamous aspect. There is considerable variation, in our series, in this regard.

Case 14 (Fig. 4*A*) is another example of this process, where, in areas of atelectasis and fibrosis, bronchial epithelium proliferates to line adjacent alveoli with cuboidal, or spindle, or squamous cells. The last two types, in turn, can proliferate to form small solid clusters and masses, which are in one case more or less squamous, in another more or less spindle-shaped. Figure 4*B*, from Case 15, shows very clearly how the proliferating cells, sneaking across the surface, show, instead of squamous cells, spindle-shaped and flattened variants, in small masses and in double and triple layers.

Prior tries to draw a distinction between a "squamous metaplasia" and the tumors that he described. Prior and Jones refuse to include a case where squamous metaplasia and proliferation go hand in hand. In our opinion the significant feature is the proliferation which fills alveoli instead of merely lining the surfaces. Whether the proliferation is squamous, with abundant metaplasia, or spindle-shaped, with little differentiation, is essentially a minor point.

We interpret two types of pathogenesis. The first derives from the bronchi and bronchioles, which have ciliated epithelium and a basal layer. The latter proliferates to form compact masses, which fill up alveoli and stream from one to another. The cells may be spindle-shaped or with some squamous differentiation. In a second type there is a proliferating bronchial epithelium, cuboidal or flattened. These cells retain considerable potentialities of growth. They line surfaces primarily, and on occasion proliferate to form solid masses. Squamous differentiation is not uncommon.

The two types of growth may coexist. Figure 5 (Case 6), from our only positive case of cystic disease, shows a low-power view of extensive proliferation. There were innumerable similar areas in this case.

We feel that fibrosis and atelectasis, giving relative fixation to the lung tissue, stimulates the growth of the epithelium of terminal bronchioles. The margins of infarcts offer the same type of stimulus, whether through fixation, atelectasis, or some more subtle influence. One may question whether there is any analogy to the epithelial proliferation and metaplasia seen near infarcts of the prostate. The greatest amount of proliferation of the entire series occurred in Case 6, that of the cystic disease. The literature shows that in the overwhelming majority of instances the atypical proliferations occur in cases of bronchiectasis, cystic disease, fibrosis, severe atelectasis, or infarction. These factors, we feel, are significant. Whether the proliferation is spindle-shaped or squamous seems not to be significant.

Are we dealing with a malignant process? Most authors consider this type of proliferation as carcinoma. Prior believes there is a similarity to bronchial adenoma. This conclusion we are not able to accept. We feel that there are two questions involved: First, are we dealing with a tumor? Second, if a tumor, is it malignant? These questions cannot be answered without a rigorous definition of tumor and malignancy, and such a satisfactory definition is not at hand. We would point out that the proliferations, by themselves, with or without the squamous metaplasia, are not necessarily neoplastic. Womack and Graham refused to call the lesions in

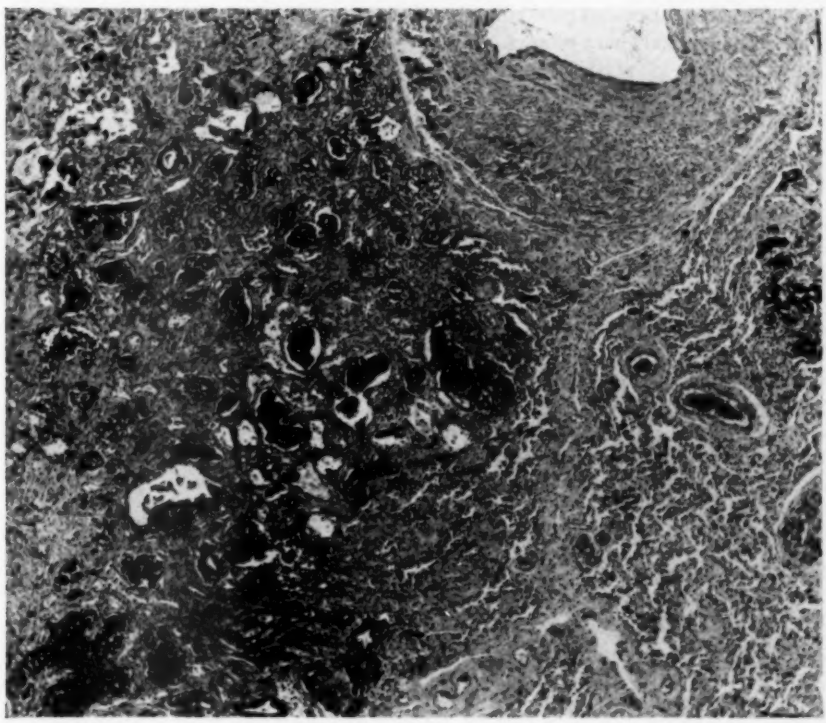

Fig. 5 (Case 6).—Case of cystic disease of the lung. Marked fibrosis on the left, severe atelectasis on the right. Low power view of cell multiplications.

their cases of cystic disease tumors. And there seems to be an essential similarity between all the reported cases, whether bronchiectatic or not. On the other hand, although we fundamentally agree with Womack and Graham, some lesions show an aggressive quality that strongly suggests kinship to a true neoplastic quality. Even if such lesions were designated tumors, we do not believe there are adequate present grounds for calling them malignant.

This type of lesion, or, perhaps we should say, these types, if we wish to make a significant distinction between the two sorts of pathogenesis, fall into that nebulous area where pathologists, for lack of accurate criteria, must hedge their bets. In other parts of the body we sometimes make the diagnosis of "atypical hyperplasia."

Such a designation in the present state of our knowledge, seems more appropriate than any other. The lesion must be considered innocent until proved guilty. The proof is not yet forthcoming, but the verdict of "not proved" does not indicate that the suspect is of upright and blameless character. Let us say that some of these atypical proliferations have the potentiality of developing into frank cancer—which ones, and why, we do not know. There are some very irritating cases in which the patient dies with widespread oat-cell type of carcinoma, suggesting pulmonary origin, but in which a primary tumor cannot be demonstrated. It is possible that some of these lesions may have developed from minute atypical proliferations of the type here described. But that possibility does not mean that all the lesions, as we observe them now, are malignant tumors. In the words of the medieval schoolmen, *in posse* is not the same as *in esse*.

SUMMARY

In 1,450 consecutive autopsies, 15 cases were found in which the lungs showed foci of cellular proliferations of atypical character. The cell masses, which fill alveoli and occasionally seem to lie within connective tissues, are generally of spindle- or oat-cell type, but sometimes of squamous character. These cells seem to derive from the basal cells of terminal bronchioles or from the familiar bronchiolar cells which grow down to line alveolar surfaces in zones of fixation. The principal predisposing factor is the fixation of pulmonary tissue, whether it be from fibrosis, or atelectasis, or immediate juxtaposition to infarcts or abscesses. The proliferations seem to be fundamentally reactive in nature, but to belong to that nebulous zone where one cannot sharply distinguish between hyperplasia and neoplasia. It is entirely possible that these proliferations may give rise, in rare instances, to carcinoma. Nevertheless, in the form in which these lesions are so frequently observed, any malignant character would be only a possibility, not an actuality.

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COMMON INFECTIOUS DISEASE OF LABORATORY RABBITS QUESTIONABLY ATTRIBUTED TO ENCEPHALITOZOON CUNICULI

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APPROXIMATELY 900 laboratory rabbits were subjected to detailed observations, including tissue sections of principal organs, from 1940 to 1953. Nearly a third of these rabbits were found to have a distinct disease syndrome characterized by systemic infection of a mild, chronic type with severer inflammation in the kidneys and brain. Most previous reports of similar naturally occurring rabbit infections have emphasized the encephalitic aspects * and, since the classic paper by Levaditi, Nicolau, and Schoen,[13] in 1924, have termed the condition Encephalitozoon cuniculi infection. Bell and Hartzell,[14] Smith and Florence,[15] and, more recently, Lewis and Thompson [16] have described a nephritis similar to that observed in rabbits with this syndrome. Nonexperimental cardiovascular changes occurring in rabbits appear to be part of this disease picture, and observations of aortitis by Kesten [17] and various reports of rabbit myocarditis † indicate lesions similar to those observed in this study. The purpose of this report is to present a unified description of the manifestations of this disease.

Hagan and Bruner [21] considered E. cuniculi infection a form of toxoplasmosis. It is likely that Toxoplasma infections have sometimes been confused ‡ with this more prevalent, mild, and chronic disease of rabbits, as stated by Perrin,§ who discussed the differentiation of Toxoplasma and E. cuniculi infections.

In efforts to reduplicate certain special stains for infectious micro-organisms within infected tissues, it became apparent that ceroid [22] or hemofuscin pigments could readily be confused with a parasitic form. It was found impossible to demonstrate E. cuniculi or any other presumably etiologic infectious agent in this disease by means of any of a variety of special stains.

MATERIALS AND METHODS

Rabbits.—From 1940 to 1946, approximately 300 rabbits were observed in Wisconsin, Maryland, and Florida; from 1946 to 1948, 153 were studied in Georgia, and since then 490 have been observed in Illinois. About 90% of the rabbits were young albinos, 2 to 6 months of age, of both

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The opinions expressed herein are those of the author and cannot be construed as reflecting the views of the Navy Department or of the Naval Service at large.

* References 1-12.

† References 18-20.

‡ References 1, 15, and 22.

§ References 11 and 12.

sexes, and purchased from commercial dealers. The breeds were heterogeneous. A few were black, brown, or gray rabbits, and occasionally there were some that were raised in the laboratory. The majority were used in experiments involving Streptococcus injury.‖

Observations.—Rabbits were observed, with frequent records of body weight and general condition, and occasional temperature readings, white blood cell counts, and blood cultures. At the conclusion of an experiment, gross pathologic changes were noted and cultures occasionally made of infected tissues. Cultures were made on blood agar and in brain-heart infusion and semisolid thioglycollate broths. Tissues for microscopic examination were fixed in formol, Zenker's fluid, 80% ethanol and 10% formol, or Bouin's fluid, and occasionally samples were placed in several fixatives for comparisons.

In addition to gross abnormalities found elsewhere, the following organs were routinely examined microscopically: brain, heart, lungs, spleen, liver, adrenals, gonads, ileum, appendix, kidneys, and femoral marrow. In many instances, the following organs were also sectioned: thymus, thoracic and abdominal aortae, stomach, bladder, spinal cord, skin, lymph nodes, and periarticular tissues. Paraffin sections were prepared and routinely stained with a heavy hematoxylin and rather light eosin stain. Special stains used were Giemsa's, Gram's, methylene blue, various carbolfuchsin preparations, Levaditi's, and various other silver stains. The method of Wright and Craighead [22] for staining supposed parasitic forms within brain lesions was altered to give better definition by employing a 0.02% basic carbolfuchsin-5% phenol solution for 18 hours, followed by a brief period in distilled water, staining in 1% aqueous methylene blue, rapid differentiation and dehydration through ethanols into xylene, and then mounting. Staining comparisons of 10% formol-fixed and Zenker-fixed portions of normal and infected tissues were made in about 200 specimens. Variously fixed human tissues were employed for certain control purposes while using special staining methods in the search for a definite infectious agent.

RESULTS

Clinical Observations on the Disease in Rabbits.—The majority of infected rabbits had a mild, chronic disease without diagnostic signs. If the infection was severe, the fur would appear roughened; the animal would be somewhat stunted and thin, and seemingly more susceptible to various noxious agents. Occasionally rabbits would have a normal gain in weight for months and appear sleek and healthy, only to present mild but definite evidence of the infection upon pathologic examination. It was not found possible to diagnose accurately the condition in the living rabbit.

Rabbits with a mild infection had normal total and differential white blood cell counts; those with a severe infection had a slight lymphocytosis. Body temperatures fluctuated widely with environmental factors and exertion and did not afford a reliable diagnostic point.

Neurologic signs were generally absent, in contrast to the motor paralysis observed by Wright and Craighead.[22] It was often surprising to find extensive cortical lesions in animals that had appeared normal, or at most had some suggestively choreiform movements consisting of erratic and poorly coordinated motions. Likewise, considerable renal infection did not result in uremic or hypertensive signs, as might be expected in human nephritis.

The impression was gained that albino rabbits were more frequently infected than Dutch and other pigmented animals. Females seemed to be a trifle more frequently infected than males; for example, in a series of 280 rabbits,[26] 30% of the males and 48% of the females were infected. Length of stay in the animal quarters was a factor; in the series of 280 rabbits mentioned, 21% were positive among those kept less than six months, while 54% were positive among those kept beyond that time.

‖ References 25 and 26.

The geographical differences encountered did not appear to make significant variations, and the geographic distribution agreed with Cowdry's report [10] on that of rabbit encephalitis. Several marked variations in the number of infected rabbits were noted in multiple purchases from the same dealer, which, however, might have been due to the custom of dealers occasionally to purchase rabbits from others when required to fill an unusually large order.

The disease appeared to be mildly contagious. Animals housed together would be more apt to have infections of this type; yet instances were observed in which rabbits sharing quarters and experimental procedures, including injections with the same needle, apparently remained free from the disease, while others were infected. A few females had a mild vaginal discharge and microscopic evidence of chronic parametritis; however, data associating Treponema cuniculi venereal infection of rabbits ¶ with this syndrome are lacking. It may be spread by venereal contact, or possibly by ear mites, as well as through infected urine from animals with active nephritic lesions. A true epidemic was not observed, perhaps owing to the mild, chronic, and asymptomatic nature of the disease. Complete pathologic study of large groups of rabbits did not reveal all to be infected.

A disease tended to smolder along and assume a chronic course, with slight remissions. Entire recovery did not occur, since foci of active inflammation were always present, even in some rabbits that were clinically improved and microscopically manifested numerous fibrotic foci of healed infection.

The infection predisposed to other natural infections and to increased injury from added experimental noxious procedures, such as the inoculation of pathogenic organisms or their toxic products. A few instances of severe carditis were observed which appeared to be due to this type of infection plus a superimposed Streptococcus infection, resulting in a progressive myocarditis, which was selfperpetuating and which somewhat resembled human rheumatic fever.[26]

Pathologic Observations.—This disease was a chronic, usually mild, and generalized infection. The cellular response was chiefly made up of the large lymphocyte or monocyte, which the rabbit characteristically has in abundance, and of plasma cells, lymphocytes, and occasional eosinophiles. Polymorphonuclear leucocytes were seldom present. There was scant stimulation, except in severely infected rabbits, of the lymphoid tissues (lymph nodes, thymus, spleen, and appendix), of the reticuloendothelial cells (splenic, liver, marrow, and lymphoid sinuses), or even of the two delicately responsive tissues, the lung and bone marrow. The alveolar phagocytic cells, which rapidly enlarge and react to infections in the rabbit, would occasionally do so in this disease if the infection was fairly extensive.

The outstandingly diagnostic feature of this disease which could be grossly observed was the characteristic nephritis. In acute infections, or those probably less than a month in duration, the kidneys were frequently enlarged, smooth, and had pale areas over the cortex. Microscopically, such kidneys manifested extensive interstitial focal nephritis with numerous foci of round-cell infiltration about peripheral tubules and similar cell aggregations about smaller arterioles. The tubules would have foci of degeneration extending to necrosis, with associated cast formation, and slight involvement of glomeruli only in that area.

¶ References 1, 2, 3, and 21.

Later on, as in rabbits manifesting some stunted growth for at least a month, this nephritis progressed to focal fibrosis. The foci of fibrosis were grossly visible on the surface of the kidney as somewhat circinate, depressed white areas about

Fig. 1.—Gross view of renal cortical scars characteristic of the described disease in rabbits; × 2.

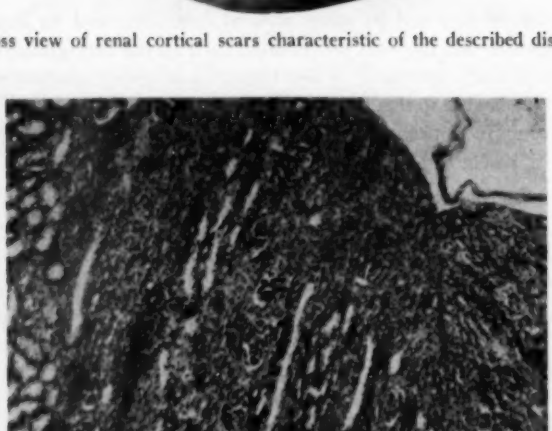

Fig. 2.—Photomicrograph of a rather large, chronic scar in the kidney, such as that seen in Figure 1; × 50.

1 mm. in width and 2 to 3 mm. in length (Fig. 1). They were fairly numerous and evenly distributed over the renal surfaces. Large, irregular scars, like those observed in pyelonephritis, were infrequent and there was no evidence of ureteral or bladder inflammation. Microscopically, such lesions still possessed numerous

Fig. 3.—Small renal scar, more commonly observed than the larger ones; × 80.

Fig. 4.—Interstitial accumulation of round cells in renal cortex extending from the surface toward the hilum; × 80.

round cells (Figs. 3 to 6), but there was increasing replacement by fibrous tissue, and occasionally fatty tissue. In older infections, round cells would often disappear from certain foci, which would be fibrotic and have obliterated tubules and glomeruli in them (Fig. 2). The report of Lewis and Thompson [16] contains numerous excellent photographs of this type of renal disease in advanced cases.

The initial lesions of the nephritis appeared to come from hematogenous rather than ascending urinary sources. There was considerable round-cell infiltration about arcuate arteries, but the necrotizing and sclerosing changes observed in human arterioles in various renal inflammations were absent (Fig. 5). Glomeruli were

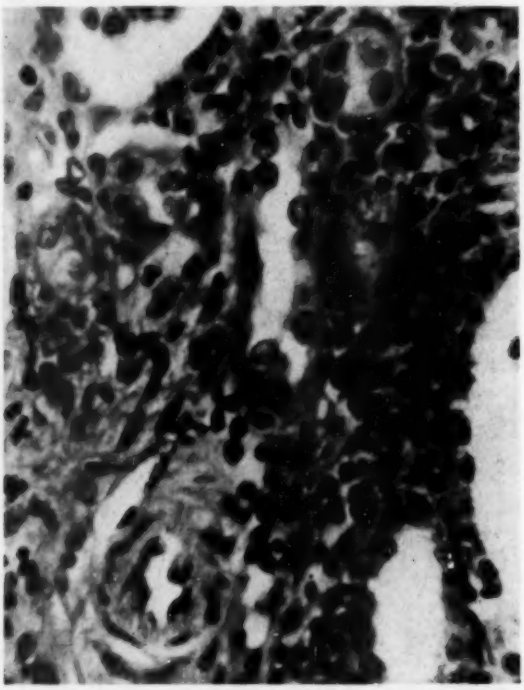

Fig. 5.—Round-cell accumulation in renal cortical area; × 475.

not primarily involved and appeared damaged only if there were extensive changes in draining tubules (Fig. 6). Foci of casts were numerous, but still these involved areas were in the minority and there remained adequate amounts of functioning renal tissue. Rabbits with only this infection did not die of uremia.

The lesions observed in the nervous system have been mentioned in numerous reports.# The inflammation consisted of cortical nectrotic foci (Figs. 7 and 8) and areas adjacent to vessels both in the cortex and in the meninges which had round-cell accumulations (Figs. 7 to 11). Sometimes there appeared only a few areas of lymphocytic infiltration amid nerve tissue degeneration, but further search revealed the characteristic small foci of degeneration with necrosis, and the round-cell peri-

References 1 through 13.

vascular cuffing. In sites of necrosis, cellular chromatin debris was prevalent, and in such tissues fixed in formol, fuchsinophilic or ceroid pigmentation [27] was present in abundance.

The cerebellum, brain stem, and spinal cord were seldom the sites of inflammation due to this disease. The cortical distribution of the lesions agreed with the paucity of motor neurologic signs other than questionable choreiform movements.

A proximal aortitis was the chief point noted in the gross pathology, in addition to the characteristic nephritic scars. This involved only the proximal portion of the aorta, and not the aortic valve or the other portions of the thoracic or abdominal

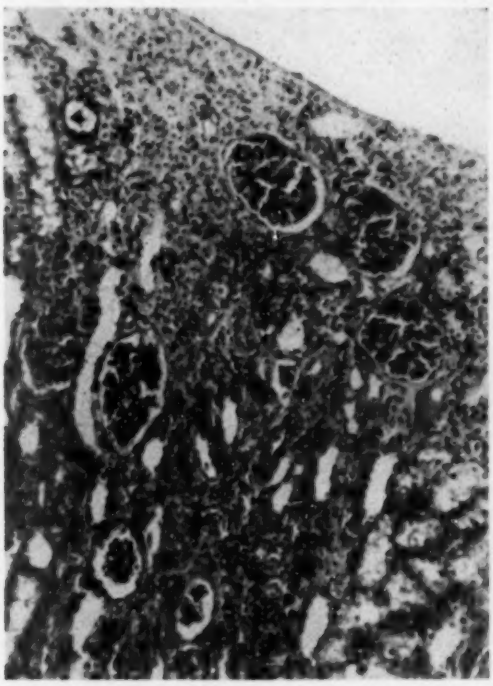

Fig. 6.—Renal cortical inflammation with extensive tubular damage but scant glomerular inflammation; × 95.

aorta. It was not as consistently present as the renal lesions, but in older animals with extensive infection was nearly always observed. It consisted of a rough, plaque-encrusted change with longitudinal striae and slight aortic dilatation. Microscopically, there was disruption of the media with associated deposition of various substances, including small amounts of calcific material, and foci of a few round cells. Occasionally the intima would have a small area of edema and degeneration with a small surface thrombus and interspersed round cells. Vasa vasorum changes were minimal, and true aneurysms were not encountered.

The arteriolar inflammation in the kidney and brain have been previously noted, and it appeared to consist of a primary arteritis. This arteritis was severest in a

few foci of coronary inflammation, especially in the papillary muscles of the left ventricle. Here there were extensive degenerative, edematous changes of the media with moderate round-cell accumulations, fibrinoid changes of the collagen, and increased numbers of bar-nucleated cells (Anitschkow's myocytes). In more chronic

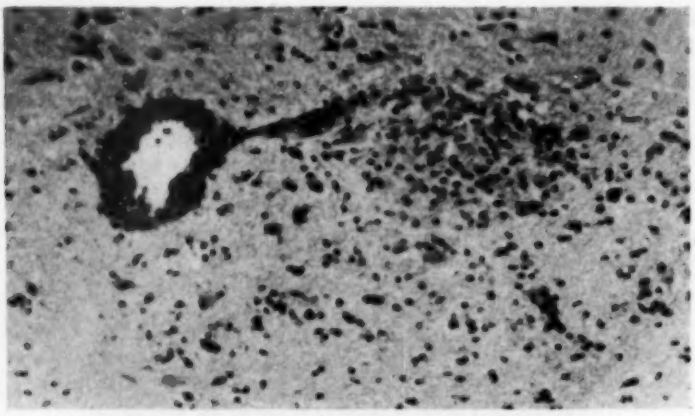

Fig. 7.—Chronic encephalitis, cerebral cortex; × 120.

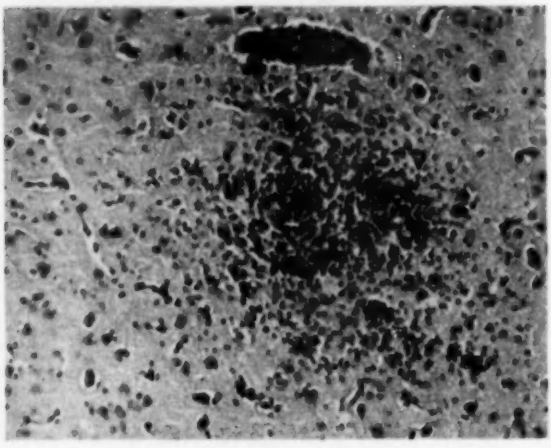

Fig. 8.—Subacute encephalitis, cerebral cortex; × 90.

lesions, extensive fibrosis occurred, with a resultant thickening of arterioles, although without typical arteriosclerotic or atherosclerotic changes. This inflammation was usually seen in only a few areas of the heart.

The myocardium offered a good diagnostic criterion for the presence of this disease syndrome by having numerous small foci of round-cell accumulations (Fig. 12) at sites of myocardial muscle injury. These foci generally were small (20 to 30 μ in diameter), often had a central area of tinctorial change suggestive of myofiber

necrosis, and consisted of densely packed monocytes, plasma cells, and lymphocytes. As elsewhere, polymorphonuclear leucocytes were rare and eosinophiles seldom present. These foci would sometimes be seen in the endocardium and pericardium, generally in a juxtavascular position. They resembled cardiac lesions of rabbits reported by others * but were not true Aschoff bodies, which Saphir and Langendorf [27] have accurately described.

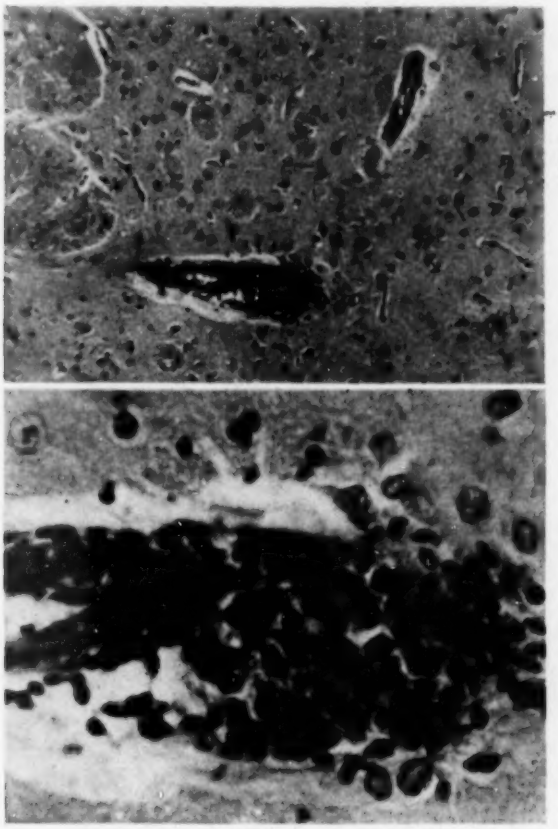

Fig. 9.—Subacute encephalitis with characteristic perivascular round cells; × 80.
Fig. 10.—Higher magnification of round cells in Figure 9; × 533.

The livers of rabbits with this disease often had increased numbers of round cells in portal foci and a slight increase in sinusoidal reticulum cells. Hepatic coccidiosis did not appear related to this infection, nor did tapeworm cysts in the mesentery. The spleen, adrenals, and lymph nodes usually were normal in size, in contrast to their enlargement in many chronic infections. The scrotum and gonads were not involved, with the possible exception of a mild, focal round-cell inflamma-

* References 18 and 19.

tion of the periuterine tissues in a few females. The testes were carefully searched for possible inflammation but were negative, and did not as a rule undergo atrophy or degeneration, as was commonly observed in other chronic infections.

Attempts to Identify an Etiologic Agent.—Cultures of blood and tissues were essentially negative for an infectious agent associated with this disease. Methods employed sufficed to isolate cocci, pasteurellae, and other common bacteria, which

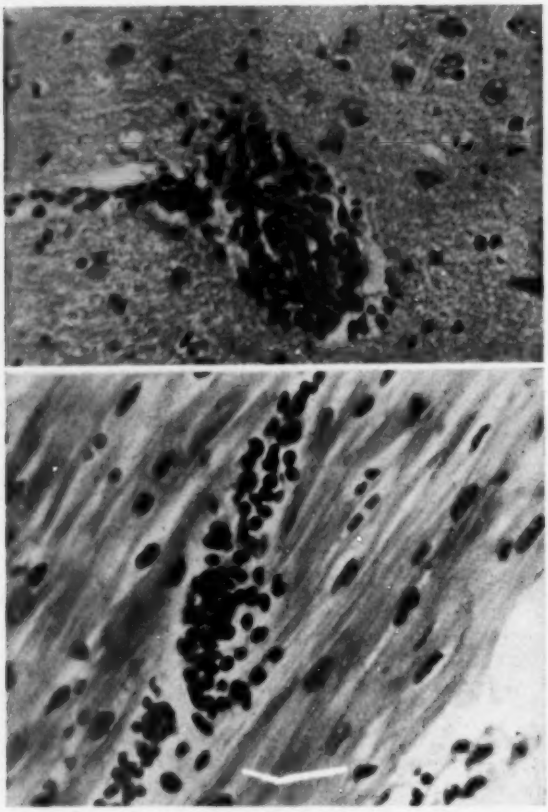

Fig. 11.—Perivascular round cells, chronic encephalitis; × 280.
Fig. 12.—Myocardial focus of round cells; × 366.

could also be clearly recognized in tissue sections. Various special bacteriologic stains likewise were negative. At sites of inflammation, especially necrotic foci in the cortex of the brain, much pleomorphic and amorphous particulate matter was present. Most of this was basophilic and appeared to be nuclear chromatin debris. In addition, tissues fixed in formol would often show fuchsinophilic granules, both within cells and lying free at sites of inflammation. The staining method modified from Wright and Craighead [22] and Goodpasture,† employing more selective absorp-

† References 9 and 29.

tion and differentiation of carbolfuchsin, proved excellent in demonstrating these red-staining forms, which had the appearance of previously reported E. cuniculi or other micro-organisms.‡ However, control materials, both human and rabbit, showed identical fuchsinophilic material associated with various inflammatory and degenerative processes. Tissues fixed in Zenker's fluid were less likely to show these materials, which appeared identical to ceroid pigment [23] or hemofuscin [24] previously shown to be present in the nervous system [27] and elsewhere [28] under various circumstances. Unstained sections showed considerable dark to yellow irregular granules associated with various types of inflammatory lesions; again, they were most prevalent in formol-fixed material and were probably hemofuscin.

A series of Levaditi, reticulum, and various silver stains were negative. The granular material appearing as the previously discussed ceroid pigmentation, and which seemed identical with some reported micro-organisms, usually stained dark with silver. Again, control materials had the same substance, and careful searches for spirochetes, Toxoplasma, or other definite micro-organisms related to this disease syndrome were negative.

COMMENT

Much of the previously reported information about this disease requires conservative and critical review, since it has been shown that fuchsinophilic materials are frequently present under various circumstances, and most likely many reported so-called E. cuniculi forms were of this nature. It is possible that occasional instances of toxoplasmosis were confused with this infection, as stated by Perrin § and by Hagan and Bruner.[21] The nuclear chromatin fragments present in areas of cell destruction could also be misinterpreted as micro-organisms. A review of Levaditi, Nicolau, and Schoen's report [18] in the light of these added data made one skeptical of their "Encephalitozoon cuniculi" parasite. It was interesting to read a similar report in the same volume designating "Encephalitozoon rabiei" as the supposed protozoan cause of rabies.[30]

This disease, with its chronic, low-grade inflammation, may be of spirochete etiology, even though various silver stains used in this study did not reveal such forms. The reasons for such a suspicion are its partial resemblance to tertiary syphilis in man; the predominating nephritis, which somewhat resembles that characterizing leptospirosis in dogs, cattle, and other animals,‖ and its possible relation to T. cuniculi infections commonly present in rabbits.¶ However, Plaut, Mulzer, and Neubürger [4] and Jaffe [1] studied this common encephalitis of rabbits from the standpoint of possible syphilitic etiology and decided it was not a manifestation of Treponema infection. It is possibly of virus etiology, although, histopathologically, it does not resemble any known virus infection. Twort and Archer [33] presumed a virus could produce nephritis in rabbits, but their data are of limited extent. Perrin's # observations that the supposed E. cuniculi agent could withstand high concentrations of glycerin and also freezing, whereas Toxoplasma could not, are open to questions of natural infections in the animal colony, and also the limited work done with rabbits.

‡ References 1, 8, 9, 11, 12, 13, 15, and 22.
§ References 11 and 12.
‖ References 1, 2, 3, 21, and 31.
¶ References 1, 2, and 3.
# References 11 and 12.

The relation of this disease to toxoplasmosis has been considerably discussed. Observations indicate a sharp difference between the two, with Toxoplasma infections assuming a fairly rapidly progressive, usually fatal infection, with prominent diagnostic signs and enlargement of the spleen and lymph nodes, containing easily demonstrated and distinct parasites. However, the question of asymptomatic toxoplasmosis is baffling, and it has been well established that a woman may remain in apparent excellent health and yet deliver an infant congenitally infected with Toxoplasma. At present it seems unjustifiable to assume variations in morphology and physiology within Toxoplasma sufficient to account for virulent and avirulent diseases, and to attribute this so-called E. cuniculi infection of rabbits to a mild form of toxoplasmosis.

Obviously, a systemic disease of the type described here would be an important factor in evaluating experimental infections or injuries. The myocarditis observed resembled the "spontaneous" myocarditis reported by others,* and which some have confused with rheumatic lesions. Additional infection with streptococci capable of causing some carditis independently resulted in severer carditis and nephritis.†

In a recently observed series of 280 rabbits subjected to various Streptococcus and Staphylococcus infections, two were observed with chronic, self-perpetuating, severe carditis.[26] One had been given alpha Streptococcus and the other a Group A Type 12 strain. Both animals differed markedly from others given the same treatments and had granulomatous lesions in the heart similar to those observed by Brown and Pearce.[20] Certain aspects of the carditis associated with this disease syndrome resembled human rheumatic fever and have elicited comment by observers,[19] who believed there was a resemblance to Aschoff bodies. These resemblances to human rheumatic fever were interesting but did not prove confusing if the strict criteria of Saphir and Langendorf [28] for true rheumatic carditis were adopted. It might be rewarding to determine the etiology of this rabbit disease, its possible synergistic relations with other infectious agents, and the part similar factors might play in the pathogenesis of rheumatic fever.

The difficulty of deciding whether a lesion was caused either by this naturally prevalent disease of rabbits or by an experimental procedure, and of evaluating quantitative relationships of multiple factors, was obviously considerable. Use of large numbers of animals within each treatment group proved the most feasible means of evaluation, since observation of the described disease syndrome in various animals scattered among differing treatment groups permitted factorial consideration. It required several years of studying numerous rabbits to comprehend clearly the specific, characteristic nature of this disease entity. Most previous reports have disproportionately emphasized the encephalitic aspects of the disease, and hence have obscured knowledge of its systemic manifestations.

It appears necessary again to emphasize the importance of considering such an infection among a group of experimental rabbits. Reports continually appear describing lesions and forming conclusions based upon data evaluated without cognizance of this disease. Experimental designs must first of all allow adequate controls, numbers of animals within a treatment group, and factorial arrangements to detect such differences; secondly, a complete pathologic study must indicate the more precise

* References 1, 18, and 19.

† References 24 and 25.

status of each animal. Gross observation of the kidneys might allow detection of over half such infections in rabbits; microscopic study of brain, kidney, and other organs should afford a certain diagnosis.

SUMMARY

Nearly a third of over 900 laboratory rabbits manifested a naturally occurring disease syndrome. It was a mild, chronic, systemic infection characterized by round-cell infiltrations. Major lesions were in the cerebral cortex, the renal cortex, and the cardiovascular system. There were no clinically diagnostic signs. Gross pathologic examination revealed characteristic small, depressed scars on the surfaces of the kidneys, and frequently inflammation of the proximal aorta. Microscopic examination of brain, kidney, and other tissues made the diagnosis certain.

Numerous previous reports have attributed various lesions of this nature to Encephalitozoon cuniculi, which some claim to be related to Toxoplasma. Special staining methods for this supposed protozoan parasite, E. cuniculi, did not reveal the infectious micro-organism but indicated that some previously reported data were confusing fuchsinophilic (ceroid) pigment with the alleged E. cuniculi. The etiology of this disease is unknown.

The relation of this disease syndrome to Toxoplasma, spirochete, and other infections is discussed and its importance to those working with experimental rabbits is emphasized.

The staff of Naval Medical Research Unit No. 4, under the direction of Commander J. R. Seal (MC), U. S. N., have aided many phases of this study from 1946 to 1953.

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ANEMIA PRODUCED BY CHLORAMPHENICOL (CHLOROMYCETIN) IN THE DUCK

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THERE are frequent references in clinical medicine to the occurrence of injury to the hematopoietic system following the therapeutic use of chloramphenicol (Chloromycetin).* The majority of the cases have shown the characteristics of an aplastic anemia.† A maturation arrest of the erythroid cells has been suggested as occurring in the cases reported by Volini and associates [5] and by Lindau.‡

In one experimental study an anemia has been observed in dogs following the administration of chloramphenicol. Smith and associates [6] in 1948 gave chloramphenicol to three dogs; two developed anemia after intramuscular injections but the third animal, given the drug orally, did not show anemia. None of the three dogs showed an appreciable change either in the total number of leucocytes or in the differential count. Gruhzit and associates [7] observed an anemia in dogs given chloramphenicol; however, it was attributed to a coincidental distemper infection. No changes in the hemogram have been observed in rabbits, mice, and guinea pigs given this antibiotic.[8]

White Pekin ducks have been used in this laboratory for hematological studies with phenylhydrazine hydrochloride,[9] selenium,[10] and malaria.[11] Preliminary observations in the duck indicated that an anemia would follow the oral administration of chloramphenicol. We are now reporting our experimental observations of the effect of chloramphenicol on the hematopoietic system of white Pekin ducks as shown by the changes in the number of erythrocytes within the peripheral blood and in the erythroid and myeloid cells within the bone marrow.

METHODS AND MATERIALS

White Pekin ducks, varying in age from 10 to 80 days, were used. These birds were kept in small groups in a battery in the duck room. Food and water were available to them at all times. One hundred ducks have been used in this study. Chloramphenicol § in capsules containing 250 mg. each was given orally. The maximum quantity given to any duck during the period of experimentation was 54.0 gm. Apparently only two birds died from the effects of chloramphenicol.

From the Laboratory of Experimental Pathology, University of Texas Medical Branch.

* References 1 to 4.

† References 1, 3, and 4.

‡ Lindau, W.: Effect of Chloromycetin upon Erythropoiesis, read before the American Federation of Clinical Research, Atlantic City, N. J., May, 1952.

§ The Chloromycetin was supplied by Parke, Davis & Company, Detroit.

The total amount of chloramphenicol per dose, per day, and per experiment, is given in the specific experiments. The drug was given only between the hours of 8 a. m. and 5 p. m.

Blood for erythrocyte counts and reticulum stains was obtained from the web of the foot. Hayem's diluting fluid and standard techniques were used for these counts. A small drop of a 1.0% solution of brilliant cresyl blue in isotonic saline was placed on a cover slip with a drop of blood for staining the reticulocytes. After the blood was mixed by pressing the cover slip onto a routine glass slide, the periphery of the slide was sealed with white petrolatum. Reticulocytes were counted after an interval of 15 to 30 minutes. The number was recorded per 1,000 erythrocytes. Red blood cells and reticulocyte counts were made on 22 ducks given chloramphenicol and on 10 normal ducks. The counts were made during both the morning and the

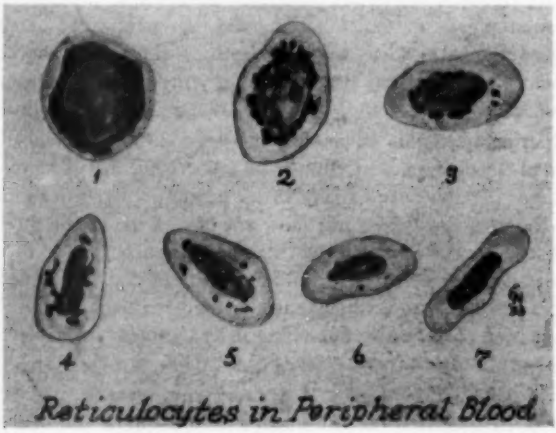

Fig. 1.—Drawing showing the decrease in the amount of reticulum that occurs as the erythroblast matures. Note that both the nucleus and the cell are round in the earlier stages and that the cell gradually becomes elliptical as it matures.

afternoon, usually for 10 days; however, in some of the birds the counts were followed for 30 days.

Reticulocytes normally show a progressive decrease in the amount of reticulum as the cells approach maturity. This variation in the amount of reticulum is illustrated in Figure 1. In one experiment the number of reticulocytes at a specific stage of maturity was counted per 1,000 red blood cells. The number of reticulocytes within the peripheral blood decreases as the duck ages. Birds 10 days of age may have 150 to 200 reticulocytes per 1,000 red blood cells, while only 10 to 15 reticulocytes are present in the peripheral blood of ducks 30 days of age. In all our experiments in which reticulocytes were counted in birds given chloramphenicol, normal birds of a similar age also were included. To obtain the best comparative results, all counts were made by the same technician.

Bone marrow for cytological study was obtained immediately after the spinal cord was severed. The marrow was aspirated from three different sites—the

femur, tibia, and humerus. The smears were air-dried and stained routinely with a combination of Wright's and Giemsa's stains. The details of this technique are to be published.[12] A total of 1,000 cells was counted from the three smears made from the three different bones. These cells were differentiated into the myeloid and the erythroid series. Bone marrow studies were made on 37 birds given chloramphenicol and on 7 normal ducks of corresponding age.

Histologic studies were made on the kidney, liver, and spleen of 20 ducks given chloramphenicol and of 6 normal ducks. These tissues were fixed in a 4.0% solution of formaldehyde and stained routinely with hematoxylin and phloxine.

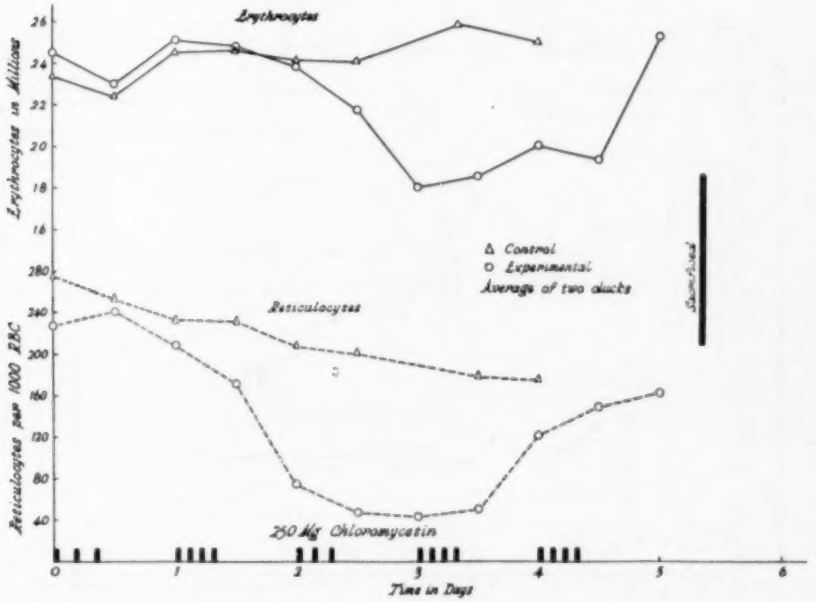

Fig. 2.—The average reticulocyte and red blood cell count on two ducks given chloramphenicol. Note the decrease in both the erythrocytes and the reticulocytes within the peripheral blood. The control is the average of two normal ducks of corresponding age.

EXPERIMENTAL STUDY

The effect of multiple doses of chloramphenicol on the total number of erythrocytes and reticulocytes in the peripheral blood is shown in Figure 2. The two ducks in Figure 3 also illustrate the fact that an anemia occurs after the oral administration of chloramphenicol. The anemia that we have observed in the duck has been only of moderate severity. Ducks 368 and 374 (Fig. 3) show the characteristic features observed in all birds with reference to the rapid decrease in the number of reticulocytes within the peripheral blood. Duck 368 also shows that the reticulocytes may completely disappear and remain absent from the peripheral blood until death. The more frequent change that occurs in the number of reticulocytes in the peripheral blood is illustrated by Duck 374. A decrease occurs during the first few days

following the oral administration of the drug, and this is followed by a return of reticulocytes in the peripheral circulation, although the bird continues to receive chloramphenicol.

The fact that the number of reticulocytes gradually decreases in the peripheral blood after the oral administration of chloramphenicol, and then spontaneously the number of reticulocytes returns to a percentage greater than normal indicates to us the need for further study. Figure 4 shows the variations in the number of reticulocytes in Duck 367 given chloramphenicol over a period of 38 days. These cells decreased in number, then rapidly increased, and finally progressively decreased to within the range of normal by the 18th day of the experiment. These observations

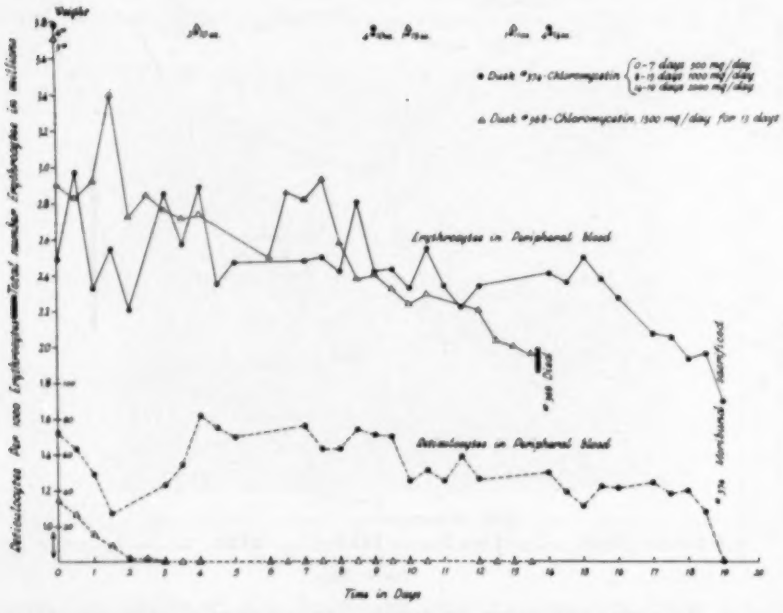

Fig. 3.—The erythrocyte and erythroblast count in two ducks given large amounts of chloramphenicol. The erythroblasts completely disappeared from the peripheral blood of Duck 368 for 10 days before death. The erythroblast count usually decreases and then increases, as illustrated by Duck 374. Anemia occurred in both birds.

suggested that probably the chloramphenicol was, in some way, affecting the erythroid cells within the bone marrow and the subsequent increase in the number of reticulocytes in the peripheral blood was being produced by activity in the extramedullary foci of hematopoiesis. Pathologic studies were made to study this possibility. No variations, however, were observed in the extramedullary blood-forming tissue in the liver, spleen, and kidney in the group of ducks receiving the chloramphenicol when compared with the controls. It should be emphasized that a morphologic study of the spleen is not satisfactory for such determinations.

Morphologic studies were made on the erythrocytes within the peripheral blood stained with a combination of Wright's and Giemsa's stains to determine whether

any changes were present within the cells. Ducks given phenylhydrazine hydrochloride have shown extensive degenerative changes in the red blood cells within the peripheral blood.[9] No changes, however, were observed in the erythrocytes of the ducks given large amounts of chloramphenicol. Since there is no evidence of a hemolytic anemia in these ducks receiving large amounts of chloramphenicol and since there does occur a rapid decrease in the number of reticulocytes within the peripheral blood, it appears to us that this antibiotic may directly affect the erythroid elements within the bone marrow of the duck.

To study this problem the reticulocytes were classified and counted with reference to age. This classification was based upon the fact that the youngest form of the

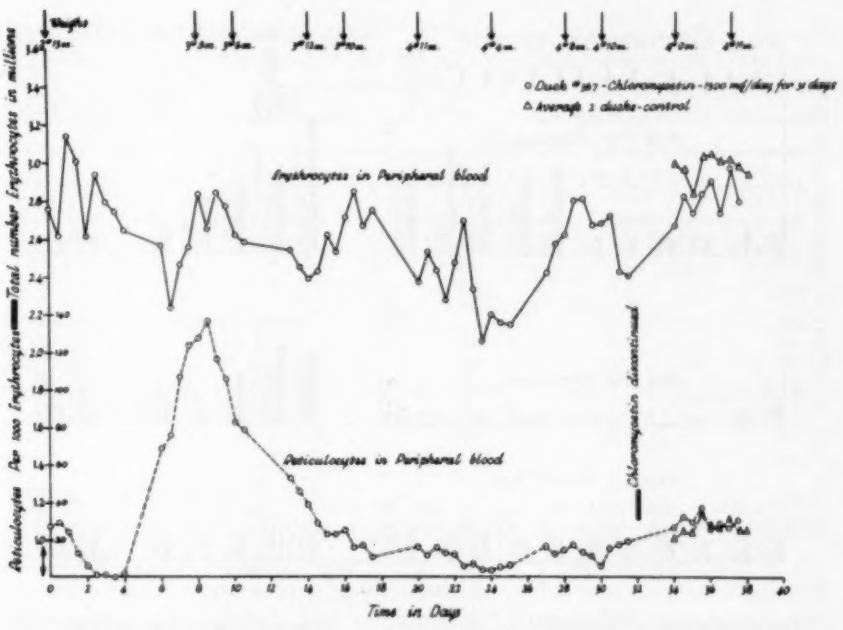

Fig. 4.—Duck 367 illustrates the usual variations observed in ducks given chloramphenicol. Note the increase in the number of reticulocytes that occurred on the fourth day. The erythrocyte and reticulocyte counts rapidly returned to normal when the drug was discontinued.

reticulocytes found in the peripheral blood is a round cell with a round nucleus. The cytoplasm stains bluish-purple, and the reticulum completely surrounds the nucleus (Fig. 1). As the reticulocyte ages, the amount of reticulum progressively decreases, and both the cell and its nucleus becomes elliptical in shape. The cytoplasm now stains pink or pinkish-red. The changes that occur in the reticulocytes within the peripheral blood as they mature are shown in Figure 1.

Four ducks of the same age were used to determine the percentage of reticulocytes per 1,000 erythrocytes illustrating three different stages in their development. Two ducks were given chloramphenicol, and two were used as controls. The results of this study are shown in Figure 5. From these data it may be noted that the

number of all stages of reticulocytes decreases in the peripheral blood of ducks given chloramphenicol. These observations would support the opinion that the anemia in the duck given chloramphenicol results either from an interference in the maturation of the erythroid cells or from an injury to these cells within the bone marrow.

The bone marrow from 37 ducks given chloramphenicol and 7 normal birds was studied. The percentage of erythroid and myeloid elements within the marrow of these ducks is given in the Table. All the ducks given chloramphenicol for three to five days have a decrease in the percentage of erythroid cells. Furthermore, these marrow smears show few degenerative changes in the erythroblastic cells. The degree of injury to these cells in the bone marrow apparently is related to the amount

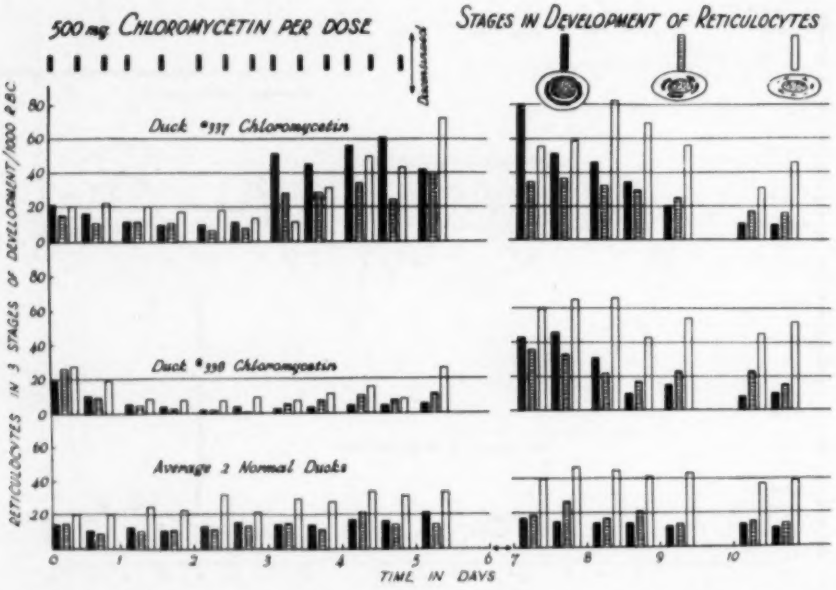

Fig. 5.—Number of reticulocytes in three stages of development per 1,000 red blood cells. Note the decrease following the administration of chloramphenicol. The reticulocytes appeared in an increased number in Duck 337 while the drug was being given. In Duck 338 the reticulocytes did not reappear in large numbers until the medication was discontinued on the fifth day. The youngest forms of the reticulocytes reappeared first when the number of reticulocytes began to increase in the peripheral blood.

of the drug given. Our data are not adequate to show whether ducks of varying age differ in their susceptibility to the action of this drug per gram of body weight. It is obvious, however, from these studies that the duck is highly resistant to the action of this drug when compared with man.

To obtain some information relative to the lethal dose of chloramphenicol for the duck, a group of 24 birds 12 days of age was used. Eight were given 500 mg. (average 1.0 mg. per gram of body weight) per day for six days; eight birds were given 1 gm. (average 2.4 mg. per gram of body weight), and eight ducks were given 2 gm. (average 4.8 mg. per gram of body weight). This dosage was determined by

the weight of the ducks at the beginning of the experiment. One-half of this dose was given at 9 a. m. and one-half at 4 p. m. None of the ducks died, and only two in the group given the largest amount lost any weight.

A sensitivity phenomenon is sometimes considered to be the mechanism by which blood dyscrasia develops in man receiving therapeutic agents. Eight ducks were given chloramphenicol for several days. After an interval of 10 to 14 days a second course of chloramphenicol was given. After a second interval, of 8 to 10 days, a third course of chloramphenicol was given. There was no evidence from these ducks that the reticulocyte and erythrocyte response was influenced in any way by multiple courses of therapy. Clinically the birds did not show any signs suggestive of a sensitivity reaction.

COMMENT

Chloramphenicol when given orally produces an anemia in the white Pekin duck. In addition to the decrease in the erythrocytes, there is a rapid diminution in the number of reticulocytes in the peripheral blood. Chloramphenicol also reduces the

Effect of Chloramphenicol on Erythroid Cells in Bone Marrow

No. of Ducks	Age, Days	Dose, Mg./K	Total Dose, Mg.	Time, Days	Percentage Erythroid Cells		Drop in Erythroid Cells, %
					Normal	Treated	
4	10	46.5	4.0	4½	56.8	26.7	53.0
4	11	46.5	4.5	5	53.9	30.5	44.0
5	11	5.6	2.75	5	53.0	36.1	31.8
5	14	5.6	2.75	5	49.7	41.5	16.4
4	17	....	1.0	4	47.6	40.7	14.4
4	21	....	2.75	8	45.8	37.0	19.0
3	24	....	3.5	7	43.0	30.3	30.2
3	24	....	4.25	12	42.7	35.4	17.0
5	24	....	3.25	4	43.3	31.5	27.0

ratio of erythroid to myeloid cells in the bone marrow. Degenerative changes occur in some of the erythroblasts within the bone marrow. The mechanism of the anemia in the duck apparently may result either from the direct injurious effect of this antibiotic on the erythroid cells in the bone marrow or from an inhibition in the maturation of erythroblasts. This mechanism differs from that of phenylhydrazine hydrochloride, since the latter injures the erythrocytes within the circulating blood, producing a hemolytic type of anemia.[9] Sodium selenite apparently interferes with the maturation of the erythroblasts within the bone marrow.[10] This effect of selenium may be prevented by cysteine.[10] Cysteine, however, does not inhibit the effect of chloramphenicol on the bone marrow.

Although the quantity of chloramphenicol necessary to produce an anemia in the duck is large as compared with that for man, it is of considerable interest to have an experimental host in which an anemia produced by chloramphenicol may be studied. Furthermore, these observations may give some indication as to the possible effect of chloramphenicol on the hematopoietic tissue in man. Additional hematological studies in the duck are indicated, since this bird may prove to be a satisfactory host for the testing of certain therapeutic agents for their effect on bone marrow.

The tremendous increase in the number of reticulocytes that usually follows the rapid decrease in the number of reticulocytes in the peripheral blood when chloramphenicol is given is interesting, especially in view of the fact that the ducks were still receiving the drug. A similar phenomenon occurred when selenium was given.[10] This increase in the number of reticulocytes may be only an overcompensatory mechanism following the anemia. The bone marrow of ducks given chloramphenicol and examined two to four days after the drug was discontinued shows an increase in the number of erythroid cells.

Adequate pathologic studies have not been made on ducks that die after the oral administration of chloramphenicol. The two birds that died during the time of this experiment showed extensive degenerative changes in the bone marrow and what appeared to be a decrease in the number of reticuloendothelial cells within the splenic sinuses. Many of the remaining cells in the spleen were pyknotic and fragmented. Similar degenerative changes were found in the bone marrow of the ducks killed during the experiments.

In view of the fact that the duck apparently is highly resistant to the effects of chloramphenicol, it would be of considerable interest to know the concentration of the drug within the blood stream. Such data were unavailable to us during the time of this experiment. The rate of absorption may vary in different species and thus account for the apparent resistance of the duck to this drug. The effect of chloramphenicol on the intestinal bacterial flora is now being studied. Preliminary observations show that the bacterial flora is not significantly affected when 750 mg. of chloramphenicol is given daily for four days. This observation would suggest that the drug may be destroyed within the craw of the duck. Such a process may account for the very large doses that were found necessary to produce the anemia.

The differential count on the reticulocytes in the peripheral blood, as shown in Figure 5, would indicate that erythroblasts rapidly mature after they reach the peripheral blood. This observation apparently would support that made in another study on the length of life of duck erythrocytes. Duck red cells when tagged with radioactive selenium show an intravascular life span of 11.7 days.[13]

SUMMARY

An anemia has been produced in white Pekin ducks following the oral administration of chloramphenicol (Chloromycetin). This anemia apparently results from the effect of this antibiotic on the erythroblasts within the bone marrow. When chloramphenicol is given, a rapid decrease occurs in the numbers of reticulocytes that are normally present in the peripheral blood of the duck.

This experimental study helps to emphasize the necessity for careful clinical observations on the hematopoietic tissue when chloramphenicol is being given.

The duck is very resistant in comparison with man to the effects of chloramphenicol.

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Laboratory Methods and Technical Notes

USE OF RITTER AND OLESON STAINING METHOD FOR DEMONSTRATION OF FUNGI IN PARAFFIN SECTIONS

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The demonstration of fungi in paraffin sections is frequently the key to a perplexing diagnostic problem. The search for these organisms is often exasperating, however, and sometimes fruitless, even in known cases of mycotic infection. This paper describes a staining method which has been shown to be exceptionally well suited to the demonstration of fungi in paraffin sections.

METHOD

Ritter and Oleson [1] have recently utilized a combination of the Hale stain [2] for acid polysaccharides and the periodic acid-Schiff (P. A. S.) reaction [*] for 1,2 glycols, including carbohydrates, to demonstrate "polysaccharides of two types" in paraffin sections. A modification of Ritter and Oleson's method has been employed for the demonstration of fungi in paraffin sections. Specimens are handled as follows:

1. Zenker's solution or Bouin's fluid is the preferred tissue fixative, but formalin is also satisfactory.

   (*a*) The fresh specimen is fixed in Zenker's solution for 24 hours and then washed in running tap water for 24 hours before immersion in 80% alcohol for 24 hours, or

   (*b*) The fresh specimen is fixed in Bouin's fluid for 24 hours and then immersed in 80% alcohol for 24 hours, or

   (*c*) The fresh specimen is fixed in 10% formalin for 24 hours.

2. After fixation, the specimen is immersed in 95% alcohol for 24 hours, then in absolute alcohol for 4 to 10 hours, and is subsequently left in toluene overnight.

3. The specimen is placed in paraffin for four hours at 50-60 C. and then imbedded in fresh paraffin.

4. Sections are cut from 4 to 6 μ in thickness, mounted on slides with glycerin-egg albumen and put in the oven at 65 C. for at least one hour.

5. The slides are immersed in xylene for two minutes to remove the paraffin and then passed through absolute alcohol and 95% alcohol to tap water. If the fixative was Zenker's solution, the slides are next placed in strong iodine solution U. S. P. (Lugol's iodine) until nutmeg-brown, then left in 5% sodium thiosulfate until bleached, and washed well in running tap water.

Read at the 103rd Annual Meeting of the American Medical Association, San Francisco, June 24, 1954.

From the Departments of Dermatology and Pathology, University of Virginia School of Medicine (Dr. Cawley and Dr. Wheeler). The Department of Pathology, University of Alabama Medical Center (Dr. McManus), and the Department of Pathology, University of Michigan Medical School (Dr. French).

[*] References 3 through 5.

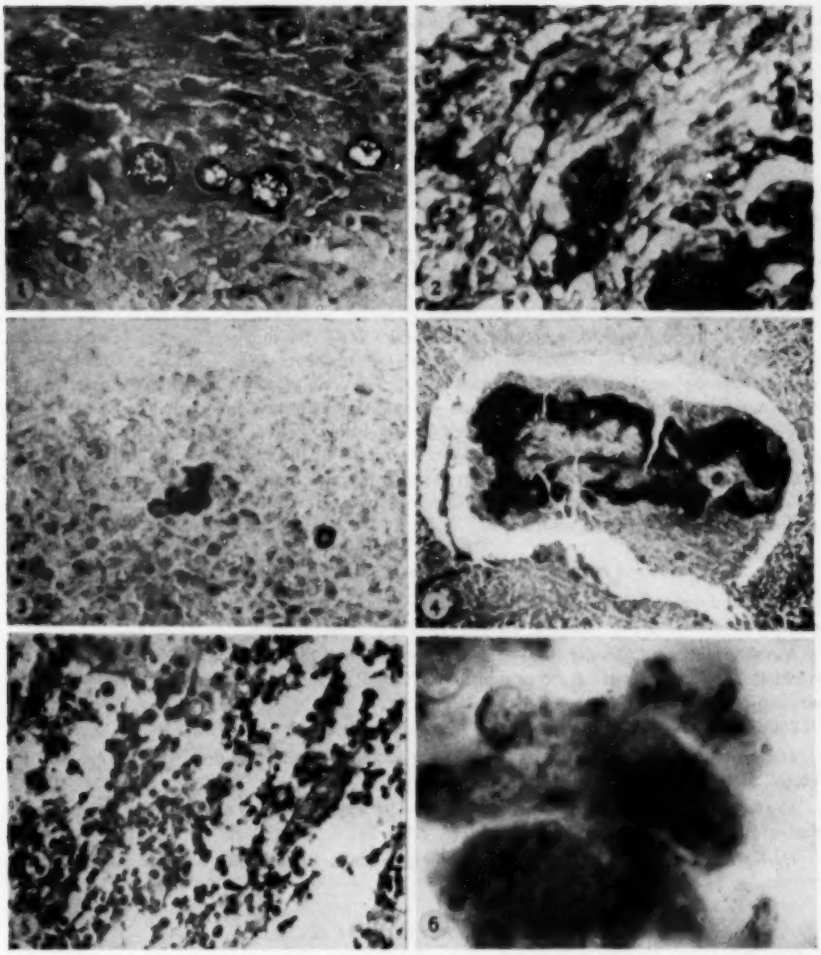

Fig. 1.—Coccidioidomycosis, showing the causative organisms in human skin. Modified Ritter and Oleson stain; × 353.

Fig. 2.—Blastomycosis, showing the causative organisms in giant cells in human skin. Modified Ritter and Oleson stain; × 353.

Fig. 3.—Chromoblastomycosis, showing the causative organisms in human skin. Modified Ritter and Oleson stain; × 353.

Fig. 4.—Maduromycosis, showing the colony in human skin. Modified Ritter and Oleson stain; × 73.

Fig. 5.—Cryptococcosis (torulosis), showing the causative organisms in human skin. Modified Ritter and Oleson stain; × 353.

Fig. 6.—Histoplasmosis, showing the causative organisms within phagocytes in human skin. Two of the organisms are in focus and stand out clearly. Modified Ritter and Oleson stain, oil immersion; × 2,166.

6. The slides are immersed for two minutes in a solution composed of equal parts by volume of dialyzed iron and 2 M acetic acid, washed well in distilled water, and placed in a solution composed of equal parts of freshly prepared 0.02 M potassium ferrocyanide and 0.14 M hydrochloric acid for 10 minutes.

7. The slides are washed well with distilled water and treated by the periodic acid-Schiff reagent method, as follows:

(*a*) Immerse in 0.5% periodic acid for five minutes.

(*b*) Wash in distilled water.

(*c*) Immerse in Schiff's reagent [4] for 15 minutes.

(*d*) Rinse in three changes of sulfurous acid, each for two minutes.[4]

(*e*) Wash in running tap water for three to five minutes.

(*f*) Dehydrate in graded alcohols, clear in xylene, and mount in balsam.

RESULTS

The histochemical specificity of the Ritter and Oleson staining method has been examined elsewhere,[1] and this investigation was concerned only with its value for the demonstration of fungi in paraffin sections. The significant colors observed in paraffin sections stained by the Ritter and Oleson method are red, blue, and green. Various shades and combinations of red and blue predominated in the sections described briefly below. These were obtained from known cases of human fungous infections and stained by means of the previously outlined modification of the Ritter and Oleson method. The color contrast between fungi and background was extraordinary, enabling prompt detection of organisms, even when small in size and few in number, with the lowest power of the microscope.

DEEP (POTENTIALLY SYSTEMIC) FUNGOUS INFECTIONS

Coccidioidomycosis.—The cell walls of the spherule and of the endospores are stained a magenta color and the cytoplasmic material of the endospores dark blue (Fig. 1).

North American Blastomycosis.—The double walls are shown to excellent advantage, as are the buds. The outer wall of the organism is stained dark blue and the cytoplasmic material purplish-red (Fig. 2).

Chromoblastomycosis.—The organism is stained a dark red color (Fig. 3).

Maduromycosis.—The central portion of the colony is stained a deep violet color and the "clubs" at the periphery bright pink (Fig. 4).

Cryptococcosis Cryptococcous (Torulosis).—The capsule of the organism is stained bright blue and the body of the organism dark red (Fig. 5).

Histoplasmosis.—The central mass of the organism is stained blue, and the pink capsule is surrounded by a dark-blue ring (Fig. 6).

SUPERFICIAL FUNGOUS INFECTIONS

The demonstration of superficial fungi in tissue sections by means of special staining techniques is seldom required because of the adequacy of simpler methods. The modified Ritter and Oleson method has also been found to be useful, however, for the demonstration of superficial fungi in paraffin sections.

COMMENT

A large number of specimens, in addition to those described above, has been examined for fungi during the past two and one-half years. These have included biopsy and necropsy material from many tissues and organs, and from a variety of cases, including known and suspected mycotic infections, as well as "unknowns." A comparison of the Ritter-Oleson, periodic acid-Schiff (the efficacy of the periodic-acid Schiff stain for the demonstration of fungi in animal tissue is well known and was first described by Kligman and Mescon [6] in 1950), and hematoxylin-eosin stain, utilizing these specimens for the collation, shows that the modified Ritter and Oleson staining method is superior to the others, in our experience, for the demonstration of fungi in paraffin sections.

SUMMARY

The Ritter and Oleson staining method, with modifications, is especially well suited to the demonstration of fungi in paraffin sections.

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News and Comment

Case History Service of American Institute of Dental Medicine.—The Institute is issuing again for the year 1953-1954, Case Histories of Dental Medicine. These will be accompanied by original 35 mm. Kodachrome slides of the oral condition, pertinent laboratory findings, medical background, roentgenograms, histologic slides, and photomicrographs.

This monthly service is available to any member of the A. D. A. or A. M. A., or their foreign equivalent.

Anyone subscribing to this Case History Service is entitled to full membership in the American Institute of Dental Medicine for the fiscal year. There are still available a few case histories for the year 1952-1953. Further information may be obtained from the executive secretary, Miss Marion G. Lewis, 2240 Channing Way, Berkeley 4, Calif.

Second Annual Symposium on Antibiotics.—The Second Annual Symposium on Antibiotics, sponsored by the Division of Antibiotics of the United States Department of Health, Education, and Welfare, with the journal *Antibiotics and Chemotherapy* will be held on Oct. 20, 21, and 22, 1954, in the auditorium of the Department, 4th St. and Independence Ave., S. W., Washington, D. C.

Dr. Jean R. Oliver Appointed Distinguished Service Professor.—Dr. Jean R. Oliver, who has recently retired as Professor of Pathology at the State University of New York College of Medicine at New York City, Brooklyn, has been appointed the first Distinguished Service Professor of the State University of New York and Professor of Pathology in the College of Medicine.

Cancer Research Program of Tobacco Industry Research Committee.—The Tobacco Industry Research Committee today invited university, hospital and other medical research organizations throughout the nation to submit proposals for specific cancer research projects for consideration by the Committee's Scientific Advisory Board.

At a meeting of the Committee's Scientific Advisory Board yesterday in New York, Dr. Clarence Cook Little, Director of the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, acting chairman, said that the Board was in the process of reviewing 60 applications and inquiries already received.

An initial fund of $500,000 for research has been appropriated by the Tobacco Industry Research Committee, which is composed of major cigarette manufacturers, tobacco growers, and warehousemen. This committee has also pledged to vote substantial additional funds for specific projects upon the advice of the Scientific Advisory Board.

Composed of seven outstanding physicians, educators, and scientists, the Advisory Board is guiding development of a long-term research program to be conducted in institutions outside the tobacco industry. In accordance with policy set when the program was announced early this year, the industry will not set up any research facilities. No industry funds will be given to tobacco company laboratories. All projects will be in addition to research carried on by companies.

Dr. Little said that the program now being developed by the Scientific Advisory Board will seek to encourage research projects into areas not now being fully explored by the 850 research grants in federal, state, and private facilities in this country.

In addition to Dr. Little, the members of the Advisory Board are Dr. McKeen Cattell, Professor, Head of Department of Pharmacology, Cornell University Medical College, New York City; Dr. Leon Jacobson, Professor of Medicine, University of Chicago, The School of Medicine, and Director of the Argonne Cancer Research Hospital, Chicago; Dr. Paul Kotin, Assistant Professor of Pathology, University of Southern California School of Medicine, Los

Angeles; Dr. Kenneth Merrill Lynch, President, Dean of Faculty, and Professor of Pathology, Medical College of the State of South Carolina, Charleston; Dr. Stanley P. Reimann, Scientific Director of the Institute for Cancer Research and Director of the Lankenau Hospital Research Institute, Philadelphia, and Dr. William F. Rienhoff Jr., pioneer lung surgeon and Associate Professor of Surgery, The Johns Hopkins University School of Medicine, Baltimore.

Sarah Mellon Scaife Fellowship in Pathology Available.—A program of fellowships designed to prepare men for academic careers in medicine, principally in the field of pathology, has been established by the Department of Pathology in the University of Pittsburgh. The fellowships will provide experience in research, teaching, and diagnostic pathology. Three years will be spent in the Department of Pathology, University of Pittsburgh School of Medicine. Arrangements will be made for one year to be spent at another medical center, of the applicant's choice.

One fellow will be appointed each year for a four-year term and will receive a stipend increasing as follows: first year, $3,000; second year, $3,300; third year, $3,700; fourth year, $4,000.

The applicant must be a graduate of an approved medical school.

Application must be made by Nov. 1 for a fellowship beginning July 1 of the following year. Selection will be made by Dec. 15.

Further information and application forms are available from Dr. Frank J. Dixon, Department of Pathology, University of Pittsburgh, Pittsburgh 13.

Deaths.—Harrison S. Martland Sr., research pathologist, author, pioneer in radioactive diseases, and retired professor of forensic medicine at New York University, New York, died May 1, 1954, at the age of 70.

Henry Bunting, associate professor of pathology at the Yale University School of Medicine, New Haven, Conn., died April 15, 1954, at the age of 43.

Awards.—The New York City Cancer Committee has presented Arthur Purdy Stout, professor of pathology at Columbia University, with the Clement Cleveland Award for "outstanding work in the campaign to control cancer" in 1953.

Ricketts Medal to Dr. Paul.—Dr. John R. Paul, of Yale University School of Medicine, New Haven, Conn., has received the Howard Taylor Ricketts Medal of the University of Chicago, given annually as a tribute to the late Dr. Ricketts, who proved during the 1906 epidemic of Rocky Mountain spotted fever that the disease was transmitted by a tick and who three years later discovered the causative organism, now named in his honor. Dr. Paul has studied outbreaks of poliomyelitis in Alaska, Japan, Egypt, and the tropics, and infectious hepatitis among the troops in Germany. He was a member of the committee on virus research of the National Foundation for Infantile Paralysis, 1940 to 1948; a consultant to the Secretary of War and director of the neurotropic Virus Disease Commission of the Army Epidemiology Board, 1941 to 1946, and chairman of the virus and rickettsial study section, Research Grant Division, United States Public Health Service, 1946 to 1951. At the award ceremony Dr. Paul spoke on infectious hepatitis.

Appointments.—Dr. James Robert Teabeaut, head of the forensic pathology section of the Armed Forces Institute of Pathology, Washington, D. C., has been made assistant professor in the Division of Pathology and Bacteriology of the School of Medicine of the University of Tennessee.

Dr. Benjamin Castleman has been appointed chief of the Department of Pathology at the Massachusetts General Hospital, and clinical professor of pathology at the Harvard Medical School. Dr. Castleman will continue his clinical research activities in the new pathology laboratory building, which is to be constructed in 1954.

INDEXES

This issue of the ARCHIVES appears in two parts, the second of which is the index for the preceding volume. To make the index more usable to the readers and to enable them to find information with less difficulty than has been encountered in previous volumes, the index has been broken down into three separate indexes: Title Index, Author Index, and Subject Index. The Title Index, which will take the place of the six-month Table of Contents, previously published with the index of the journals, has been prepared in such form that it can be separated from the other indexes and bound in the front of the volume. It consists of the titles of the articles arranged in alphabetical order and accompanied by the name of the author, the name of the senior author if there are more than two, the names of both authors if there are two, and the page number on which the article begins. The Author Index contains the names of all authors of articles appearing in the ARCHIVES and of the presenters of cases or papers before societies. The third index, the Subject Index, contains in alphabetical order the important subjects which have been dealt with in the volume. Individual abstracts are no longer indexed; only the categories under which they have been classified are listed. Book Notices and news items are included in this Index.

Books

Mayo Clinic Diet Manual. By Committee on Dietetics, Mayo Clinic. Second Edition. Price, $5.50. Pp. 247. W. B. Saunders Company, 218 Washington Sq., Philadelphia 5; W. B. Saunders Company, Ltd., 7 Grape St., London W. C. 2, 1954.

This second edition, in loose leaf form, contains diets, and directions for their use, as these have been found of value at the Mayo Clinic. Particular attention is given to diets to be used postoperatively. In the appendix many tables supply information needed daily by dietitions and physicians in the planning of therapeutic diets of various sorts. The printing and indexing will make it easy to use by those concerned with problems of nutrition.

Cardiovascular Surgery. By Gerald H. Pratt, M.D. Price, $15.00. Pp. 843, with 358 illustrations in 261 figures and 4 color plates. Lea & Febiger, 600 S. Washington Sq., Philadelphia, 1954.

The purpose of this text is to give surgeons, internists, and students a summary of acceptable therapy for cardiovascular lesions. The subject matter is comprehensive, including major sections on surgery of the heart, arteries, veins, and lymphatics, and other sections dealing with related topics, such as anesthesia, cardiac arrest, angiography, skin grafting, portal hypertension, and the role of isotopes in vascular disorders. Each of the main sections includes chapters on every disease of the vessels under consideration. The organization is good, and any particular topic may be located easily. Excellent illustrations are abundant, and the printing is good. The proofreading lacks care, as shown by numerous errors in spelling.

This publication's chief value will be as a reference for the student and practitioner. Because of its encyclopedic nature, many topics are inevitably covered in a brief fashion and without the care and authority to be expected in a more limited monograph. Its greatest strength lies in those sections dealing with the practical therapeutic aspects of vascular disease, where the author's experience is wide. The book may be useful for a survey of the field by students and as a review or an introduction to specialized vascular topics for others.

INTERNATIONAL Hemacrit

A *New* HIGH-SPEED MICRO-HEMATOCRIT CENTRIFUGE FOR CAPILLARY BLOOD TUBES

Rapidly increasing recognition of the time and labor saved by the improved Guest-Siler method for rapid, accurate, blood cell volume testing has prompted the introduction of the new International Hemacrit Centrifuge. The new technique involves four rapid steps: 1. Blood sample is taken directly from finger in a heparinized capillary tube. 2. Tube is sealed. 3. Sample is centrifuged 4 or 5 minutes at 11,000 r.p.m. 4. Per cent blood cell volume is read directly by means of a reader or microscope. Results are accurate, the test can be made quickly, only a micro quantity of blood is necessary, and the inexpensive micro-capillary tubes can be used once and discarded, eliminating the cleaning of expensive glassware. See "Journal of Laboratory and Clinical Medicine," Vol. 19, No. 7, April, 1934.

The International Hemacrit Centrifuge is designed specifically to spin 24 of the glass capillary blood tubes at speeds in excess of 11,000 r.p.m. Its many exclusive features make it the centrifuge of choice for this application.

SAFETY — The high-speed rotating head is completely enclosed, insuring protection to the operator.

ACCURACY — The 24 tube slots in the head are individually numbered for easy identification of blood samples.

CONVENIENCE — When the protective dome is opened, the head is completely exposed for ready accessibility. The unique design of the head permits easy removal of the glass tubes.

SPEED — The quiet, dependable universal motor is capable of maintaining a minimum of 11,000 r.p.m. on any 110 volt A.C. or D.C. circuit.

AUTOMATIC — A simple twist of the knob turns the centrifuge on and at the same time sets the running time. Centrifuge shuts off automatically.

BEARINGS — The motor has sealed bearings which are lifetime lubricated and require no attention.

STABILITY — The weighted base insures stability, and rubber suction feet eliminate creeping.

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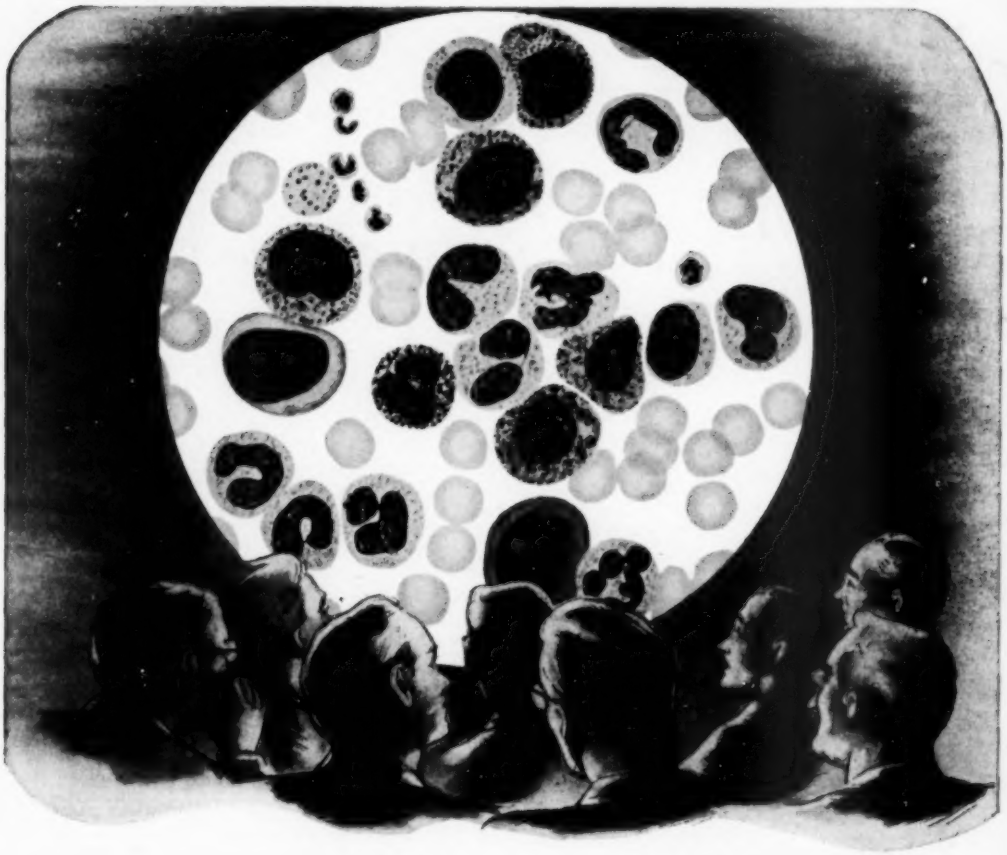

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